In vitro cloning of *Chrysanthemum paludosum* – an important ornamental plant.

Dissertation submitted in partial fulfillment of the requirement of the degree of

MASTERS OF SCIENCE IN BIOTECHNOLOGY

Under the guidance of:

Dr. Manju Ananad

Associate Professor

Submitted by:

Nikita Asija

Roll no. 301201011

DEPARTMENT OF BIOTECHNOLOGY

THAPAR UNIVERSITY, PATIALA

July, 2014

CERTIFICATE

This is to certify that the thesis entitled "***In vitro* cloning of *Chrysanthemum paludosum* – an important ornamental plant"** submitted by Nikita Asija (301201011) in partial fulfilment 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 other degree or certificate in this or any other University or Institute.

Manju Anand 18/7/14

Dr. Manju Anand
(Supervisor)
Associate Professor,
Deptt. of Biotechnology,
Thapar University, Patiala.

Dr. Dinesh Goyal
Professor and Head of Department,
Department of Biotechnology
Thapar University, Patiala.

Dr. S.K. Mahopatra
Dean (Academic Affairs)
Thapar University, Patiala.

CANDIDATE'S DECLARATION

I hereby declare that the work presented in the thesis entitled "***In vitro* cloning of *Chrysanthemum paludosum* – an important ornamental plant**" in partial fulfilment of the requirements for the award of Degree of Masters in Science in Biotechnology to Thapar University, Patiala is my own work during the period of January 2014 to June 2014, under the guidance of Dr. Manju Anand, Associate Professor, Department of Biotechnology, Thapar University, Patiala. I have not submitted the matter embodied in this report for the award of any other degree.

Date: 18 July, 2014.

Nikita Asija
Roll No. 301201011

It is certified that the above statement made by the student is correct to the best of my knowledge and belief.

Date: 18.7.14

Dr. Manju Anand
Associate Professor
Department of Biotechnology,
Thapar University,
Patiala.

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Date:

Nikita Asija

(301201011)

Abbreviations

BAP	Benzylamino purine
MS	Murashige and Skoog's medium
NAA	Naphthalene Acetic acid
IBA	Indole 3-butyric acid
IAA	Indole 3-acetic acid
Kn	Kinetin
µM	Micromolar
°C	Degree Celsius
°F	Degree Fahrenheit

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ABSTRACT

The current investigation was carried out on an important ornamental plant *Chrysanthemum paludosum* belonging to family Asteraceae. Various vegetative parts like nodal segments, shoot apices, stem segments and leaves were excised from a healthy and mature field grown plant. The different explants were surface sterilized and inoculated on Murashige and Skoog's medium (1962) supplemented with various growth regulators for multiple shoot proliferation, callus induction and *de novo* adventitious shoot and root formation.

Chrysanthemum paludosum exhibited good degree of multiple shoot proliferation from nodal explants. Multiple shoot proliferation was observed on MS medium supplemented with BAP (4.44 - 17.76 µM) or Kn (4.65 – 18.6 µM) in conjunction with lower concentrations of NAA (2.68 – 5.37 µM). Amongst these various combinations, best results were obtained on NAA (2.685 µM) + Kn (4.65 µM) where 20-22 shoots were formed from a single axillary bud.

The plants exhibited high degree of propensity for *de novo* adventitious root and shoot formation directly from the stem and leaf segments. Prolific *de novo* root formation from leaf explants was observed on different concentrations of NAA, IBA and IAA, whereas direct root formation from stem segment occurred only on 22.84 µM of IAA. Roots formed on surface of explants bore dense root hairs while those growing inside the medium did not bear any root hairs. Different hormones of different concentrations had varied effects on morphology of roots formed.

De novo adventitious shoot formation was observed directly from the stem and leaf segments. From leaf explant, *de novo* shoots were regenerated on MS + NAA (5.37µM) + Kn (4.65µM) with maximum of 12-15 shoots after 4 weeks. Out of various combinations tried, *de novo* shoot formation occurred on MS medium supplemented with Kn (4.65 µM) and lower concentrations of NAA (2.85 – 5.71 µM) and IBA (2.45 – 4.9 µM). From stem segments, de novo shoot regeneration occurred on IBA (2.45 – 19.6 µM) + Kn (4.65 µM) with maximum of about 8-10 shoots.

Callusing from stem and leaf segments occurred on MS medium variably supplemented with plant growth regulators in different combinations. MS medium supplemented with Kn (4.65 µM) and higher concentrations of NAA (21.48 µM) or IBA (19.6 µM) was found to be the best for callus induction from leaf and stem segments respectively..

The calli obtained from leaf and stem segments varied slightly in morphology. All the calli were heterogeneous and composed of cells of various shapes and sizes. The calli also showed significant histogenetic differentiation in form of tracheids that possessed reticulate thickenings on their walls.

The calli formed from stem segments on IBA (2.45-4.9 µM) + Kn (4.65 µM) exhibited high frequency shoot differentiation from the callus. The leaf callus exhibited only rhizogenic differentiation on MS + NAA (10.74 µM) + Kn (4.65 µM) after six weeks of culturing forming long, thin and slender roots.

INTRODUCTION

Plants have formed part of the human existence since time immemorial. The immortal connection between man and nature has flowered into a profound human appreciation of plants as objects of beauty and works of art (Middleton and Vosloo, 2011). A variety of values can be attributed to plants. Amidst the speedy urbanization of world, plants very often provide an important link with the natural world. Ornamental plants bring aesthetic, physical and psychological enhancements to our surroundings and add economic value to them. Moreover the ornamental plants offer a virtually limitless gene pool from which "novel" target genes can be extracted (Middleton and Vosloo, 2011).

FLORICULTURE – AN OVERVIEW

Flowers have always remained an integral part of social fabric of human life. Flowers symbolize honour, purity, beauty, peace, hope, love and passion. Flowers are commonly viewed as highly symbolic and they promote self esteem and satisfy the need of aesthetically pleasing surroundings (Krech et al., 1969; Shodhganga, 2010).

The usage of flowers and foliage for personal and ceremonial use has seen recorded to prehistoric times (Simpson & Ogorzaly, 2001; Middleton and Vosloo, 2011). Throughout history flowers have found their special place in celebration of special occasions of the moment and beautiful means for expressing emotions. Man has traditionally used flowers for worship and religious offering to God and Goddesses, complimenting the beloved ones and versifying any conceivable emotion. Floral ornaments, bouquets and floral arrangements form the pride of social gatherings. The arrival of newborn is rejoiced with flowers, the sick are wished speedy recovery by offering flowers while the dead are bidden farewell with flowers along with the tears of sorrow. Even those who dismiss floriculture as a luxury cannot afford to do without flowers when the occasion comes (Randhawa, 2004).

Floriculture is "a discipline of horticulture concerned with the cultivation of flowering and ornamental plants for gardens and for floristry, comprising the floral industry" (Getu, 2009). Floriculture is no longer in the plant and flower business. Rather it is in the fashion, decorating, lifestyle, well-being and emotion communication business (Shodhganga, 2010). Floriculture has

provided new ways to fuse art, science, nature and life into unique synthesis of expression, while conveying to the beholder a heightened awareness of nature's fragility. Gardens enjoy a unique status among the other forms of art, because of the living, earthy and spatial qualities they bring to our daily reality (Miller 1993: 178; Middleton and Vosloo, 2011).

Floriculture is an ancient farm activity with immense potential for generating remunerative self-employment among small and marginal farmers. Flowers are cultivated for aesthetic purposes as also for their fragrance, perfumes and medicines. Changing life style of people has led to the commercialization of flower cultivation. The huge demand for flowers coinciding with various occasions has led to growth of market for flowers. Cut flowers are one of the most globally produced commercial mass production items (Kendirli and Cakmak, 2007). Floriculture is becoming a booming industry in the World today (Shodhganga, 2010). Most developing nations which have geographic advantage take it as a solution to achieve rapid economic growth (Kravanja, 2006).

GLOBAL FLORICULTURE TRADE:

Floriculture is a booming industry today but competitive one. Growing at a compounded annual growth rate (CAGR) of 15%, the Global floriculture industry is likely to cross Rs 9 lakh crore mark by 2015 from the current level of about Rs 6 lakh crore (ASSOCHAM – The Associated Chambers of Commerce and Industry of India). There are currently, over 120 countries that are active in floriculture production on a large scale. The world trade of floriculture is dominated by Netherlands, United States, Japan, Italy, France, Germany and Canada, in terms of production value of cut flowers and plants. Europe, USA and Japan share the global floriculture market as leading consumers of floriculture products (Shodhganga, 2010). The organization of International Floriculture Trade, to a large extent is situated along the regional lines. Asia-Pacific countries are the principal suppliers to Japan and Hong Kong. African, Middle Eastern, and other European countries are the dominating suppliers to Europe's main markets. Colombia and Ecuador lead the market in the USA (ITC, International Trade Centre, 2014).

The worldwide annual consumption of commercially grown cut flowers has been estimated to range from US$ 40 - 60 billion. While worldwide consumption has been on the rise, consumers have also become more refined in demanding new products. To meet this growing and changing demand, production has continued to move from countries that have traditionally been consumers and growers, such as the Netherlands, to other relatively new producing countries such as Israel, Colombia, Ecuador, Kenya, and Ethiopia (ITC, International Trade Centre, 2014).

Europe has been counted as one of the highest density region of flower production per hectare (10% of total world area and 44% of world flower and pot-plant production) (European Commission, Agriculture and Rural Development). New production centres stretch from Africa to Asia and Australia. East African countries like Ethiopia, Kenya and Tanzania can now be enumerated as important floriculture industries. In Latin America, the lead hosting flower cultivators include Colombia, the second largest exporter of flowers in the world, and Ecuador. India, Israel, Thailand and Malaysia are notable flower exporters in Asia. Australia and New Zealand, because of their location within the southern hemisphere, supply seasonal flowers that are otherwise unobtainable in North America and Europe (African Business, 2014).

FLORICULTURE IN INDIA

Floriculture in India is developing as a high growth industry and a booming export sector. Government of India has acknowledged the potential of the floriculture industry and has accorded it 100% export oriented status. It comprises the florist trade, nurseries, bulb and seed production, micropropagation material and extraction of essential oils from flowers (Muthukumaran, 2008). India's floriculture industry is growing at a compounded annual growth rate of about 30%. Currently, the floriculture industry in India is poised at about Rs 3,700 crore with a share of a meagre 0.61 per cent in the global floriculture industry which is likely to reach 0.89 per cent by 2015 (Sandeep and Bapat, 2012).

Exports of floricultural products have been growing at a CAGR of 15 percent over the past decade. Indian floriculture industry is now shifting its focus from traditional flowers to cut flowers for export purposes. The country had exported 22,485.21 million tonnes of floriculture products to different nations worldwide of worth about Rs. 455.90 crores in year 2013-14. Major export destinations in 2013-14 were United States, Netherlands, Germany, United Kingdom, United Arab Emirates, Japan and Canada. Agricultural and Processed Food Products Export Development Authority (APEDA), is working for export promotion and development of floriculture in India (APEDA, Govt. of India, 2014).

Availability of varied agro- climatic conditions in different regions of India enables the cultivation of almost all kinds of flowers and favours the floriculture production potential of country. Floriculture production is concentrated largely in Maharashtra, Karnataka, Andhra Pradesh, Haryana, Rajasthan and West Bengal (Sundram, 2012). Tamil Nadu is the leading loose flower

producing state whereas West Bengal leads in the production of cut flowers in India (Muthukumaran, 2008).

Rose is the primary cut flower grown all over the country. Indian farmers grow more than 60 varieties of rose against 168 varieties grown across the globe. Other valuable international cut flower crops in the country are Chrysanthemum, Gladiolus, Tuberose, Asters, Gerbera, Carnations, Lilies, and Orchids (Sandeep and Bapat, 2012).

The area under cultivation of flowers is increasing rapidly. In 2012-13, approximately 232.74 thousand hectares area was under cultivation of flowers. The production of loose and cut flowers has also shown increasing trend in past decade. According to the 2012-13 statistics, the production of loose flowers was estimated to be 1.729 million tonnes and 76.73 million tonnes of cut flowers (APEDA, Govt. of India, 2014).

Plant Tissue Culture of Ornamental Plants

In vitro culture is one of the important tools of plant biotechnology that exploits the totipotency character of plant cells, a concept proposed for the first time by Haberlandt in 1902. The maintenance of living material by traditional method is expensive, laborious and risky. Clonal propagation through tissue cultures offers an alternative to vegetative practices used in the past and has the potential to provide high multiplication of uniform genotypes, resulting in short term gains (Beck *et al*., 1998; Kavitha *et al.*, 2012).

Tissue culture has become a routine technique in agricultural and horticultural development which has revolutionized the ornamental industry. The techniques for *in vitro* propagation of ornamental plants and tissue culture laboratory equipment are being continuously improved to meet the demand of the floriculture breeding and industry (Rout *et al*., 2006; Hesar *et al*., 2011).

Many commercial laboratories, international and national institutes worldwide use *in vitro* culture system for rapid plant multiplication, germplasm conservation, elimination of pathogens, genetic manipulations and for secondary metabolite production. The range of routine technologies has expanded to include somatic embryo- genesis, somatic hybridization, virus elimination as well as the application of bioreactors to mass propagation (Idowu, 2009). The technique focuses on improving various characteristics to enhance ornamental values, including flower colour, size and form, and production quality. Through *in vitro* micropropagation, the increasing demand of product

can be met easily and the technique also offers an opportunity to develop new germplasm and conservation (Rout *et al*., 2006).

Advantages of Micropropagation

1. Production of very large number of high quality propagules within a short time span.
2. Production of disease – free plant material with the possibility of eliminating viral, bacterial and fungal contamination.
3. Production of a large stock of true-to type clonal propagation material.
4. Long-term *in vitro* conservation of germplasm.
5. Selection and generation of transgenic plants.
6. Possibility of bringing newly bred plants and selections to market quickly and in large quantities.
7. Production and maintenance of pathogen-free stock plant material.
8. Use of only small amount of original germplasm.
9. Plant material can be marketed all the year round, even during off-season.
10. Useful in multiplication of sterile plants or plants that produce very less seeds.
11. Micropropagation often produces more robust plants that show accelerated growth.
12. Production of secondary metabolites.

Techniques of Micropropagation:

Three basic techniques are used for *in* vitro propagation of plants.

1. **Multiplication by apical and axillary bud proliferation:**

Micropropagation by apical and axillary buds is considered to be most applicable and reliable method for shoot proliferation.

Shoots proliferate by release and growth of pre-existing apical and lateral buds/meristems in the initial culture. Apical dominance is overcome by incorporating cytokinins in the growth medium. Meristematic cells are uniformly diploid and genetic changes are least susceptible, therefore, the technique ensures genetic stability of clones.

2. **Multiplication by *de novo* adventitious shoot proliferation:**

Adventitious shoots arise:

- Directly from plant part (explant) other than from preformed meristems (apical or axillary buds).
- Indirectly from unorganized callus obtained from explant.

New adventitious shoots can develop directly from explants like root, stem, leaf, petiole, flower parts, etc. Plants produced by organogenesis from callus often show cytological abnormalities. Therefore, adventitious shoot formation directly from explant is surely a better approach than the callus method for commercial clonal propagation of plants.

3. **Somatic Embryogenesis:**

   It involves the formation of bipolar embryos from the somatic cells which can develop into fully functional plants under appropriate conditions. Differentiation and organization of a somatic embryo can take place either directly from the explant or from callus. This depends on the type of explant, composition of culture medium and subculture regime.

Stages of Micropropagation

Micropropagation procedure is divided into different stages. Murashige and Skoog (1962) proposed three (I to III) stages. Debergh & Maene (1981) added stage ’0′. Currently we have accepted five stages procedure (0 to IV). Each stage has its own requirements.

Stage 0: Preparative stage

This stage involves stock plant selection and its preparation to provide quality explants. The mother plant is grown in glasshouse or under hygienic conditions to avoid the risk of contamination in further stages.

Stage1. Initiation and Establishment of sterile culture

This is the most critical stage determining success.

It includes:

- Isolation of explant – almost any plant tissue/organ can be used but shoot apices and axillary buds are considered best for commercial micropropagation.
- Surface sterilization – explant is treated with various disinfectants in multiple courses to remove contamination.
- Establishment on an appropriate culture medium – sterile media supplemented with sources of energy, minerals and hormones.

Stage 2: Multiplication of shoots and their elongation

Cytokinins are generally incorporated in the media to stimulate shoot multiplication.

The shoots are produced from explant via any of the following pathways:

- Multiplication by proliferation of axillary buds
- Multiplication by proliferation of adventitious buds
- Multiplication by organogenesis from callus
- Multiplication by somatic embryogenesis from callus

The shoots produced can be multiplied for an indefinite period by repeated subculturing.

Stage 3: Pretransplant: Induction of rooting in shoots generated *in vitro*

Shoots generated in stage 2 are transferred to rooting media (media supplemented with auxins). This step prepares the shoots for transfer to soil.

Stage 4: Transfer of plantlets to natural environment (acclimatization):

- Adaptation of plant to gradual changes in its environment when transferred from controlled culture conditions to natural environment.
- Plants produced under controlled conditions – constant temperature, high humidity, low light – need to be acclimatized when transferred to field conditions.
- Plants grown under cultural conditions have:
    1. Poor photosynthetic efficiency
    2. Uncontrolled transpiration
    3. Heterotrophic mode of nutrition

- Various changes to be brought during acclimatization are:
    1. Shift from heterotrophic to autotrophic mode of nutrition
    2. Development of photosynthetic apparatus
    3. Development of functional stomata

To achieve these changes, transplanted plantlets are initially maintained at high relative humidity (90%) for few days and then gradually lowering the humidity, plantlets can be successfully acclimatized. Similarly, during initial days light intensity is kept low and then plantlets are gradually moved to higher light intensities to promote vigorous growth (Bhojwani and Razdan, 1996).

RATIONALE AND OBJECTIVES

Chrysanthemum paludosum is conventionally propagated by seeds and by vegetative propagation via cuttings. But, both these methods have many drawbacks. Plants propagated by seeds show high genetic variability. Germination percentage and germination rate of seeds is also not so high to fulfil the increasing market demand of chrysanthemum. Vegetative propagation by stem cuttings is also not a desirable option as there is high risk of bacterial and fungal contamination. Big percentage of plants grown by vegetative means is not disease-free. Growth of plants by conventional methods is slow and season – dependent. Climatic variations influence the conventional growth of plant to major extent.

To avoid this, *in vitro* micropropagation is a highly acceptable and reliable method for propagation of *Chrysanthemum paludosum.*

The main objectives of this study are:

- To develop a reliable protocol for rapid and large scale propagation of plants in short duration of time and space.
- To obtain genetically pre elites.

No research work has been reported yet on *in vitro* cloning of *Chrysanthemum paludosum.*

REVIEW OF LITERATURE

German botanist Gottlieb Haberlandt, regarded as "Father of Plant Tissue Culture', turned the first milestone in path of science and art of plant tissue culture and gave the idea of totipotency. Micropropagation is based on the concept of totipotency and can be regarded as the miniature version of clonal propagation carried out under controlled and aseptic cultural conditions. Micropropagation has now come to stay as an important technique and has been commercialized globally for the rapid production of a number of commercially important plants like *Chrysanthemum*, *Gladiolus*, *Fressia*, *Gerbera*, carnations, lilies, eucalyptus and many other important fruit trees and medicinal plants.

The demand of cut flowers and various other floriculture products has increased enormously in past years. Technique of *in vitro* propagation has helped the breeders to great extent to meet the increasing demand of floriculture industry and overcome many agronomic and environmental problems which was otherwise not possible through conventional breeding methods.

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

1. Multiplication by apical and axillary buds.
2. Multiplication by adventitious shoots.
3. Somatic embryogenesis.

The first two approaches involve multiple stages to produce complete plantlets. These include: unipolar shoot formation which are multiplied followed by rooting of the grown multiple shoots. In contrast to this, third approach i.e. somatic embryogenesis which involves the formation of bipolar embryos which form complete plantlets by passing through stages similar to that of zygotic embryos.

All the above mentioned techniques have been successfully implemented for the production of ornamental plants under *in vitro* conditions.

Multiplication by Apical and Axillary buds:

Multiplication by apical and axillary buds is the most reliable and desirable method for *in vitro* mass propagation as this method is simple, straight forward and ensures genetic stability of the clonal plantlets generated because the cells of meristematic tissue are uniformly diploid and least susceptible to genotypic aberrations. Apical and axillary buds contain quiescent or active meristems depending upon the physiological state of explant and when grown under high concentrations of cytokinins, usually develop into axillary shoots. These shoots divide further to increase in number and can be subcultured indefinitely to raise thousands of plants from a single bud.

Karim *et al*. (2002) reported multiple shoot proliferation from nodal segments and shoot tips of *Chrysanthemum morifolium* on MS medium supplemented with 1.0 mg/L BAP reporting maximum response of 95% and 91 % respectively. He also reported that BAP was more effective as compared to Kinetin in shoot regeneration from the explant and the proliferation efficiency of nodal segments was significantly higher as compared to shoot tips. Waseem *et al*. (2009) reported maximum shoot initiation (93.3%) of *Chrysanthemum morifolium* from shoot tip explant on MS medium supplemented with BAP (1.0 mg/L). Also, intermediate levels of BAP (1.0 mg/L) in conjunction with lower concentrations of IAA (0.1 mg/L) showed good results. Waseem *et al*. (2011) developed an efficient protocol for plant regeneration from the nodal segments of *Chrysanthemum morifolium* on MS medium augmented with IAA (0.3 mg/L) producing 80.0 % shoot initiation and an average of 4.0 shoots per explant. Nalini (2012) reported MS medium with Kinetin 3.0 mg/L + IAA 2.0 mg/L to be the best combination for regeneration of multiple shoots from shoot tips of *Chrysanthemum morifolium* with 67.82 per cent success in 17.91 days.

Hedge *et al*. (2011) established a protocol for *in vitro* propagation of *Rosa* using shoot tip and nodal explants on MS medium supplemented with 2 mg/L BAP + 0.2 mg/ L Kinetin + 25 mg/L adenine sulphate where maximum number of shoots per explant obtained was 25-30, with nodal explants showing better response over shoot tips. Mahmoud *et al*. (2011) reported sucrose concentration of 2% to give significantly better results than 3% of sucrose for multiple shoot proliferation in *Rosa hybrida*. 100 % shoot proliferation from nodal segments of *Rosa hybrida* was reported on MS medium containing 2.0 mg/L BAP and 0.1 mg/L NAA by Maurya *et al*. (2013).

Kaviani *et al*. (2011) presented an efficient protocol for micropropagation of *Matthiola incana* - an important ornamental plant - using shoot tips on MS medium supplemented with 2 mg/L Kn. Kharrazi *et al*. (2011) reported 2mg/L BAP + 0.2 mg/L NAA supplemented MS medium to be best suited for *in vitro* shoot multiplication of *Dianthus caryophyllus* from axillary buds. Multiple shoot proliferation from apical buds in *Gladiolus grandiflorus*, an ornamental plant on MS medium supplemented with 0.5 mg/L IBA and 2 mg/L BA was reported by Haouala *et al*. (2012). Multiple shoot proliferation was also reported in D*endranthema grandiflora* from axillary buds on modified Murashige and Skoog's (MS) medium supplemented with BA (0.1mg/L) and GA_3 (0.5 mg/L) with a proliferation rate of 3.2 new microshoots per explant by Kereša *et al*. (2012). Markovic *et al*. (2013) reported high rates of multiple shoot proliferation from shoot tip and nodal cuttings of *Dianthus deltoids* on MS medium containing 0.1 mg/L of BAP and 0.1 mg/L of NAA.

Multiplication by Adventitious shoots:

Multiplication by adventitious shoots is also a popular and widely accepted method for successful in *vitro propagation*. It involves two methods: direct organogenesis i.e. new adventitious shoots develop directly from the explants (stem, leaf, petiole, floret, etc.) without involving any callus formation and indirect method involving formation of callus from these explants and differentiation of callus to form new adventitious shoots. This method is efficient but prone to genetic instability.

Indirectly through callus:

Bhattacharya *et al.* (1990) established a procedure for indirect micropropagation of *Chrysanthemum morifolium* from leaf and stem callus on MS medium supplemented with 0.1 mg/l IAA and 0.2 mg/l BAP. Mandal *et al*. (2000) reported callusing of stem segments of *Chrysanthemum morifolium* on MS medium with 1.07 µM NAA + 9.29 µM Kn and development of shoot buds in 50% calli forming about 6 shoots/responding callus. He also reported callus induction in floret explants of *Chrysanthemum morifolium* with high frequency of shoot bud formation on MS medium supplemented with 23.23µM Kn + 5.37 µM NAA. Vantu (2005) reported indirect micropropagation of *Chrysanthemum morifolium* from stem segments on MS medium with 2mg/L BAP and 0.2 mg/L NAA.

Indirect shoot regeneration from leaf and stem calli of *Dianthus caryophyllus* was reported by Kanwar and Kumar (2009) on MS medium supplemented with 2 mg/L thidiazuron (TDZ) and zeatin singly or in conjunction with NAA or IAA. Garcia *et al*. (2011) reported indirect organogenesis from callus derived from stem and leaf segments of *Passiflora suberosa* with highest regeneration efficiency from stem segments cultured on MS medium containing 44.4 µM BA. Zhang *et al*. (2011) established a novel protocol for callus-mediated shoot regeneration of *Curcuma kwangsiensis* using shoot base sections excised from *in vitro* seedlings as explant source on MS medium supplemented with 1.4 µM TDZ, 17.8 µM BA and 2.7 µM NAA producing an average of 8.2 shoots per callus. Reddy and Bopaiah (2012) reported regeneration of plantlets from callus derived from leaf of *Anthurium scherzeriaum* on MS medium supplemented with BAP (1.0 mg/L) and NAA (0.5 mg/L).

Directly from the Explant:

Lee *et al*. (1997) developed a reproducible protocol for plant regeneration in *Chrysanthemum coronarium* by regeneration of adventitious shoots from leaf discs at high frequency (73%) on MS medium containing BA (2.5 µM) and NAA (2.5 µM). Further addition of 1 mM $AgNO_3$ was found to enhance shoot regeneration from the explant. Song *et al*. (2011) reported 100% shoot induction from leaf, petal, petiole, and stem segments of *Chrysanthemum morifolium* on MS medium supplemented with 6.66 µM BA, 8.56 µM IAA and 0.46 µM Kn. Among different explants tested, highest frequency of shoot organogenesis was reported from the petal explants.

Naing *et al*. (2014) reported shoot regeneration directly from leaf segments of the *Chrysanthemum* cv. Vivid Scarlet on MS medium supplemented with 1 mg/L of BA + 2 mg/L of NAA under light conditions without any initial dark period.

Tymoszuk and Zalewska (2014) reported *in vitro* regeneration of adventitious shoots from ligulate florets of *Chrysanthemum grandiflorum* on MS medium supplemented with BAP or Kn and NAA.

Somatic Embryogenesis:

Somatic embryogenesis involves somatic cells of non-zygotic origin from which complete plantlets can be generated. It is associated with the formation of a bipolar structure where both shoot and

root meristems are seated and this structure proceeds to maturity in a manner similar to zygotic embryos.

Ilahi *et al*. (2007) reported rapid clonal propagation of *Chrysanthemum* through embryogenic callus formation from nodal explants on MS medium augmented with BAP. The callus exhibited various stages of embryo development and formed normal plants. Mani and Senthil (2011) reported indirect somatic embryogenesis in *Chrysanthemum* via callusing from leaf explant and petal explants on MS medium containing 1.5 - 2.0 mg/L 2,4-D. Xu *et al*. (2012) reported high induction frequency of embryogenic callus from leaf and stem explants of C*hrysanthemum* (cv. Yuukou) on MS medium supplemented with BA 1.0 mg/L + NAA 1.0 mg/L.

Keresa *et* al. (2012) reported somatic embryogenesis from leaf, stem and petiole explants of *Dendranthema grandiflora* on MS medium supplemented with 1 mg/L NAA or 2,4-D, 0.1 mg/L BA, 200 mg/L casein hydrolysate (CH) and 290 mg/L proline, with maximum response from leaf explant. Naing *et al*. (2013) developed an efficient protocol for induction of somatic embryogenesis and plantlet regeneration from petal explants of *Chrysanthemum morifolium* (cv. Baeksun) on the MS medium supplemented with 1.0 mg/L 2,4-D and 3.0 mg/L BA. In addition, 1% sucrose was also reported to be the best suitable for induction of secondary embryogenesis. Naing *et al*. (2013) reported somatic embryogenesis from leaf explants of *Chrysanthemum* cv. Euro on MS medium supplemented with 2.0 mg/L 2,4-D and 2.0 mg/L Kn, yielding a high number of embryos.

As far as *Chrysanthemum paludosum*---the present experimental material is concerned, no investigation on micropropagation through any of the techniques has been reported in the literature so far.

MATERIAL AND METHODS:

CHOICE OF MATERIAL:

Chrysanthemum paludosum was chosen as the experimental material. It belongs to family Asteraceae. It is commonly known as Creeping Daisy, Mini Margueritte, White Buttons, Snow Daisy (Fig. 1).

Commercial chrysanthemum cultivars are globally important cut flower and pot plant species. The name "chrysanthemum" comes from the Greek words – 'Chrysos' meaning golden and 'anthos' meaning flower. Chrysanthemum is ranked second, next only to rose in importance among the floriculture crops in the world. It is grown for its aesthetic as well as commercial value. A large number of cultivars of chrysanthemum are grown worldwide and they exhibit wide variation in respect of growth habit, size, colour and shape of blooms that make the chrysanthemum an excellent flower crop (Ahmad, 2011).

DISTRIBUTION:

Chrysanthemums were first cultivated in China. They are native to Asia and North - Eastern Europe and Mediterranean regions. Most species originate from East Asia and the centre of diversity is in China. There are about 40 valid species. Chrysanthemums are now widely distributed in frost free regions of world. Major countries include Germany, Japan, Australia and US. In India, Karnataka is the most prominent chrysanthemum growing state followed by Tamil Nadu, Andhra Pradesh and Maharashtra. (Ahmad, 2011)

MORPHOLOGY:

Chrysanthemum paludosum is a compact, low-growing, semi-hardy, herbaceous perennial plant. The inflorescences constitutes small daisy like flowers. Flowers are white and yellow in colour. White ray-shaped petals are arranged around the yellow centre buttons (Fig. 2). Flower size ranges from 1 – 1.5 inches in diameter. The plant shows quick blooming of flowers – about 12 weeks. The plant is a liberal self – sower with spreading habit. The plant has sharp pinnate foliage. Leaves are thick, coarse, leathery, succulent and deeply toothed. The color of leaves varies from medium to dark green.

The plant is easy to grow from seeds. Seeds must be sown 8-12 inches apart for solid coverage. *Chrysanthemum paludosum* grows about 9 - 12 inches in height and has a mounding, trailing habit that spreads to 15 inches.

CULTIVATION:

SOIL:

Sandy loam soil with good texture and aeration and rich in organic matter are best suited for chrysanthemum plants. The pH of soil must be maintained within range of 6.5 – 7.0 (slightly acidic to neutral). Soil, compost and cocopeat in ratio 1:1:2 is also considered as a good combination for growth of chrysanthemum plants. Soil must be well drained until the seeds have germinated. For blooming plants moderate soil moisture is sufficient. Soil rich in phosphorus fertilizer gives bigger and brighter flowers. Very light, sandy soils are not recommended because of their poor moisture-holding capacity.

TEMPERATURE:

Seeds germinate well in soil at temperature: 60 – 65 ^{o}F. Day temperature between 20 °C and 28 °C and night temperature of 15 °C to 20 °C during the early days of bud formation is highly desirable.

High temperatures may cause wilting of the plants, delay the production schedule, reduce floral quality, reduce summer-time productivity and cause some buds not to open at all.

LIGHT:

Light quality and duration supplied during the daily photoperiod affects the flowering response of chrysanthemum. Long day conditions during vegetative stage (upto 4-5 weeks from planting) and short day conditions during flower bud initiation stage are preferred by chrysanthemum plants. Chrysanthemums grow best in full sun, but the blooming flowers last longer if they are not in direct sunlight.

HUMIDITY:

Seedlings must be watered regularly until they become established. Chrysanthemums require a good amount of water to maintain optimum health but they must not be overwatered as too much moisture will cause disease and root rot. Water supply should be reduced as the plants approach the flowering state. Relative humidity of about 70 - 85 % is suitable for chrysanthemums.

VENTILATION:

Chrysanthemums need plenty of air circulation. Chrysanthemums should be planted in areas with adequate cool air. Proper ventilation and airflow are critical to growth of plants as they support gaseous exchange between plant and nature (Agriculture, Forestry and Fisheries Department, Republic of South Africa, 2011).

USES:

Flowers of *Chrysanthemum paludosum* are perfect for border fronts and short flowerbeds. They are used as filler in potted flower arrangements. The trailing quality adds grace to hanging basket. The white of *C. paludosum* is a good choice in mixed plantings, cooling down hot colours and intensifying dark ones. These flowers symbolize innocence, simplicity, and modesty. These plants attract bees, butterflies and resist deer.

Extracts of C*hrysanthemum paludosum* have been shown to have anti – bacterial activities against various bacterial strains and anti fungal activity against yeast. Methanolic extracts of C*hrysanthemum paludosum* have also shown the presence of flavonoids (Sassi *et al.*, 2008).

Culture Vessels:

Generally, vessels used to culture plant material include borosilicate or pyrex glasswares. The glassware used for experimental work included conical flasks (100ml, 150ml, 250ml, 500ml and 1,000ml), culture tubes (25 × 125mm), culture bottles (8 × 3 inches), graduated measuring cylinders (100ml and 1,000ml), Petri dishes, beakers and a range of pipettes (1ml, 2ml, 5ml and 10ml). Glassware was thoroughly cleaned before use. It was first cleaned with detergent and water and then with hot chromic acid ($K_2Cr_2O_7 + H_2SO_4 + H_2O$) followed by thorough rinsing with tap water. All glassware was then steam sterilized.

Cotton plugs were made out of absorbent surgical cotton wrapped in muslin cloth. Plastic caps were also used to plug the culture tubes.

Culture Media:

Plant tissue culture media is an artificial nutrient supplement consisting of organic nutrients, inorganic salts, a carbon source, vitamins and plant growth regulators. The Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) which is a classical basal medium used worldwide for plant tissue culture was used as a nutritional medium.

Stock solutions of major, minor and organic elements were made and stored at -4°C in cold room. Stock solutions of generally 4X major elements, 1000X minor elements, and 100 X organic constituents were prepared. These stock solutions were stored at 4^0C and were used within 15- 20 days. Stocks of hormones were also made either1X, 2X or 4X. They were also kept at -4^0C.

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TABLE 1 . Composition of Murashige and Skoog's (MS) 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
FeSo4.7H2O*	
Minor Elements	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	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

*Ferric Na EDTA is the alternative to the use of these two salts and is added freshly to the medium (i.e. 0.04 gm/L).

Sucrose	20000 (2%)
Agar – agar	10000 (1%)

At the time of media preparation, pre-determined amounts of these stock solutions were added in sequence and volume makeup was done with distilled water. $FeSO_4$ – EDTA, sucrose and growth regulator and were added in sequence to the basal medium. pH of medium was adjusted to 5.8 with 0.1 N HCl or NaOH. 1% agar (gelling agent) was added to the media and heated till boiling. 25 ml of media was then poured into each glass tube and plugged tightly. The media was autoclaved at 15 lbs/in^2, 121°C for 20 minutes. Test tubes were placed over racks that tilt the test tubes during cooling and gave slanted surface to the agar medium.

Following supplements were used either singly or in combination for the morphogenetic studies:

i. Basal Medium
ii. BM + NAA, IBA and IAA (1-4 ppm each)
iii. BM + NAA (0.5-4 ppm) + Kn (1 ppm)
iv. BM + IBA (0.5-4 ppm) + Kn (1 ppm)
v. BM + NAA (0.5 ppm) + BAP (1-2 ppm)
vi. BM + BAP (0.5-4 ppm)
vii. BM + Kn (0.5-4 ppm)

Surface sterilization of inoculum:

The explants like leaves, stem, apical and axillary buds were cut from the source plant material. Explants were surface sterilized before inoculating them into the media. They were put in glass jars covered with net at mouth and were washed thoroughly under running tap water for 30 minutes to remove all the contaminants like dust particles and microbes adhering to the surface of explant. This was followed by treatment of explants with liquid detergent (teepol) for 15 minutes for and then thorough washing with tap water to remove the detergent. The explants were then treated with 0.1% bavistin solution (fungicide) for 20 minutes and thereafter washed thoroughly with sterile distilled water to completely remove the fungicide.

The explants were then transferred to the sterile hood and surface disinfected with 0.1% $HgCl_2$ solution for different time intervals (2-3 min) depending upon the type of explant and finally rinsed with sterile distilled water for 3-4 times to remove $HgCl_2$. The stem explants were trimmed at both the ends to remove dead tissues, prior to inoculation.

Inoculation:

The transfer of surface sterilized explant to media is carried out under strict aseptic conditions in laminar flow hood. Before starting, hands were wiped with 70% alcohol. Material like scalpel, forceps, glass plate were autoclaved beforehand. All the material required for inoculation, except explant, and the floor and walls of laminar hood was thoroughly wiped with spirit to maintain aseptic conditions. Alcohol was sprayed with atomizer in the laminar cabinet. Ultraviolet light was switched on for 60 minutes to decontaminate all the material. Sterile practices were followed to transfer the explant into media vessel. The media vessel was resealed after inoculation inside the laminar hood to avoid contamination.

Culture conditions:

All the cultures were maintained in an air conditioned room at a temperature of 25±4°C. The source of illumination consisted of 4 feet wide fluorescent tubes (40W) fitted on all shelves where cultures are placed. The intensity of illumination was 50µm m-2s-1 lux at the level of cultures and 16 hour light regime was followed by 8 hours of darkness.

RESULTS AND OBSERVATIONS:

Different vegetative explants like nodal segments, leaves and stem were taken from a healthy field grown plant. After sterilization they were planted on variously supplemented Murashige and Skoog's medium for induction of multiple shoots from nodal segments, *de novo* root and shoot formation, callus induction and organogenetic differentiation from leaves and intermodal segments.

Nodal segment or Axillary bud Culture:

Nodal explants, each holding a lateral bud were collected from the mother plant. After sterilization, both ends of explant were trimmed off to remove any dead tissue. Nodal segments 3-4 mm in length were cultured on MS medium supplemented with various growth regulators.

Axillary shoot proliferation from nodal explant was tested on various growth regulators used either singly or in combination with each other. Best results were obtained on MS medium supplemented with NAA + Kn thereby showing a definite synergism between the two. On MS medium supplemented with NAA (2.685µM) + Kn (4.65µM), initial bud break occurred after 12 days of inoculation (Fig. 3) forming 4 – 5 shoots from axillary position after about 21 days (Fig. 4). The shoots multiplied further forming 10-12 shoots after 4 weeks of inoculation (Fig. 5). Number of shoots increased to 20-22 after 6 weeks (Fig. 6). The length of shoots increased only to some extent but well developed leaves were formed.

Similar results were obtained from MS medium supplemented with NAA (5.37µM) + Kn (4.65µM). Bud break initiated after 15 days of inoculation forming 10-12 shoots after 6 weeks (Fig. 7). The shoots did not increase in number but only moderate increase in length of shoots was observed.

As the concentration of NAA increased, the tendency of shoot proliferation decreased and that of callus induction increased. On MS medium supplemented with NAA (10.74µM) + Kn (4.65µM), callusing and shoot proliferation were observed simultaneously. (Fig. 8) However, the extent of shoot proliferation was very less.

On MS medium supplemented with NAA (21.48µM) + Kn (4.65µM), only callusing of nodal explant was observed and the medium did not support any shoot proliferation.

Response of various concentrations of NAA and Kinetin on multiple shoot proliferation from nodal segments is depicted in Table 2. and Figure 9.

Table 2

MS medium + GRS	**Time period**	**No: of shoots formed**
NAA (2.68µM) + Kn (4.65 µM)	2 weeks	0
	3 weeks	5
	4 weeks	12
	6 weeks	22
NAA (5.37 µM) + Kn (4.65 µM)	2 weeks	0
	4 weeks	12
	6 weeks	12
NAA (10.74 µM) + Kn (4.65 µM)	2 weeks	0
	4 weeks	5

Leaf Culture:

Leaf segments 6-8 mm in length were excised from source plant material and inoculated on MS basal medium supplemented with different growth regulators.

Direct *de novo* adventitious root formation:

Leaf explants exhibited a high propensity of rooting on Murashige and Skoog's medium supplemented with different auxins like NAA, IBA and IAA.

On NAA (5.37µM) supplemented medium, rooting from the explant occurred after 25 days forming 4-5 roots from the cut ends (Figs. 10 & 11). Roots were thin, long and whitish green in color (Fig. 12). On 10.74 – 21.48 µM NAA, rooting was observed earlier i.e. after 15 days and the length and number of roots increased with each passing week. On NAA (10.74µM), roots were thick and whitish brown in color (Fig.13) whereas on NAA (21.48µM), nearly 24 roots were formed which were dark green in color (Fig. 14).

Highest number of roots were, however, formed on IBA (4.9µM), where approximately 30 roots were formed after 14 days (Fig. 15). The roots were thin, long, whitish green in color. On higher concentrations of IBA (9.8µM & 19.6µM), roots formed were thick, green and brown in color (Figs. 16 & 17).

IAA induced rooting much faster as compared to NAA and IBA forming roots after 5-7 days.

On IAA (5.71µM), roots formed were small, whitish and some of them having prominent root hairs (Fig.18). On IAA (11.42µM), roots were long and greenish (Fig. 19). On IAA (22.84µM), the number of roots formed was much higher and the morphology of roots was different from others. Roots were very thick and short (Fig. 20).

Effect of various concentrations of different auxins on root formation from leaf segments is depicted in Table 3. and Figs. 21 & 22.

Table 3.

MS medium + GRS	No: of days for root initiation	% Rooting	No: of roots formed
NAA			
5.37 µM	25 days	20%	4
10.74 µM	15 days	40%	13
21.48 µM	15 days	20%	24
IBA			
4.9 µM	14 days	30%	30
9.8 µM	14 days	30%	26
19.6 µM	14 days	40%	20
IAA			
5.7 µM	7 days	80%	10
11.42 µM	7 days	100%	9
22.84 µM	7 days	90%	25

Direct *de novo* adventitious shoot formation:

MS basal medium supplemented with NAA (5.37µM) + Kn (4.65µM) favored direct adventitious shoot formation from the leaf explants. Regeneration of shoots occurred 20 days after culturing of explants. 4-5 shoots were formed initially (Fig. 23) and the number increased to 12-15 after 4 weeks (Fig. 24). The shoots were green in color and increased in length with passage of time.

MS medium supplemented with higher concentrations of NAA (10.74 – 21.48 µM) in conjunction with Kinetin (4.65µM) supported callusing of leaf explant and no adventitious shoot formation was observed.

Callus induction:

Callusing of leaf segment occurred on MS medium supplemented with NAA (10.74 – 21.48 µM) + Kn (4.65µM). Best growth of callus occurred on 21.48 µM NAA + 4.65µM Kn, when callusing started along the entire surface of leaf explant after 2 weeks of culturing (Fig.25). After 4 weeks the entire segment turned into a mass of green, soft and friable callus (Fig. 26).

Extent of callus induction from leaf explant on different concentrations of NAA and Kn is depicted in Table 4.

Table 4.

Hormone Composition	Explant	Callusing
NAA (2.68 µM) + Kn (4.65 µM)	Leaf	+
NAA (10.74 µM) + Kn (4.65 µM)	Leaf	++
NAA (21.48 µM) + Kn (4.65 µM)	Leaf	+++

(+ - Callusing to lesser extent; ++ - Callusing to moderate extent; +++ - Callusing to higher extent)

Study of Callus

The callus was soft and friable. It was heterogeneous in nature comprising of cells of different sizes and shapes like round, ovoid and elongated. Cells invariably had starch grains scattered throughout the cytoplasm.

Differentiation:

Xylogenesis:

Four week old leaf callus revealed differentiation of tracheids which occurred in groups. Some tracheids were highly elongated (Fig. 27) while others were elliptical, ovoid and isodiametric and possessed reticulate thickenings on their walls (Fig. 28).

Rhizogenesis:

Callus formed on NAA (10.74 µM) + Kn (4.65 µM) showed differentiation of roots after 6 weeks of inoculation. Several thin, long, slender and greenish white roots were formed from the callus (Fig. 29).

Caulogenesis:

No shoot differentiation was observed from the callus on various media combinations tried.

Stem Culture:

Stem segments, 5-6 mm in length were inoculated on MS basal medium augmented with different plant growth hormones in different concentrations.

Direct *de novo* adventitious root formation:

Prolific rooting was observed from the stem segments both along the entire surface and at the cut ends on MS medium supplemented with IAA (22.84 µM) alone. Initially several white roots having dense root hairs regenerated from the explant after one week (Fig. 30). Two types of roots were observed; roots growing above the medium were thin with dense root hairs and roots growing into the medium were thick, clustered and invariably devoid of root hairs (Fig. 31). Initially, these roots were green in color but with passage of time they became brownish (Fig. 32).

Direct *de novo* adventitious shoot formation:

Direct adventitious shoot formation from stem explants occurred on MS medium supplemented with IBA (2.45 – 19.6 µM) + Kn (4.65 µM) in 20% cultures.

On MS medium supplemented with IBA (2.45 µM) + Kn (4.65 µM), shoot bud initiation occurred directly from the explant after 8-10 days of inoculation (Fig. 33) forming 8-10 shoots after 18-21 days. A moderate increase in length of shoots was observed forming well developed green leaves (Fig. 34).

Similar results were obtained on MS medium supplemented with IBA (4.9 µM) + Kn (4.65 µM), where numerous shoot buds initiated after 10 days of inoculation (Fig. 35) forming 7-8 shoots after 20 days of inoculation. Shoots were short in length and yellowish green in color (Fig. 36).

In many cultures callus formation occurred simultaneously with *de novo* shoot formation. Figure 37 shows a cluster of adventitious shoots regenerated at one end of the stem segment along with callusing at the other end.

Callus Induction:

Callusing of stem segment occurred to different extent on MS medium supplemented with IBA or NAA along with Kn or BAP. However, best callusing was observed on IBA (19.6 µM) + Kn (4.65 µM), where callusing started from the cut end of stem segment (Fig. 38) which transformed into a mass of yellowish green and compact callus after 5 weeks (Fig. 39).

Effect of various growth regulators on stem segment for callus induction and adventitious shoot proliferation is depicted in Table 5.

Table 5.

Hormone Composition	Explant	Advent. Shoot	Callusing	Organogenesis
IBA (2.45 µM) + Kn (4.65 µM)	Stem	++	+	++
IBA (4.9 µM) + Kn (4.65 µM)	Stem	+	+	++
IBA (9.8 µM + Kn (4.65 µM)	Stem	-	++	+
IBA (19.6 µM) + Kn (4.65 µM)	Stem	-	+++	+
NAA (2.68 µM) + BAP (8.88 µM)	Stem	-	++	-
NAA (10.74 µM) + Kn (4.65 µM)	Stem	-	+	++

(+ - Callusing to lesser extent; ++ - Callusing to moderate extent; +++ - Callusing to higher extent)

Study of Callus

Stem callus was yellowish green, very hard and compact and thus had to be teased repeatedly with needle to study its cell types. Cells were heterogeneous in nature and showed wide variations in size and shape. Cells contained numerous starch grains (Fig. 40).

Differentiation:

Xylogenesis:

Four week old callus revealed histogenetic differentiation of callus in form of tracheids. Tracheids were present in compact groups (Fig. 41) and possessed reticulate thickening on their walls (Fig. 42).

Rhizogenesis:

No root differentiation was observed from the callus.

Caulogenesis:

High frequency shoot differentiation from the callus was observed on MS medium supplemented with IBA (2.45 – 19.6 µM) + Kn (4.65 µM). 80% of explants callused showing differentiation of numerous shoots. Initially, clusters of small shoots differentiated from the callus after 7-8 weeks from all over the callus (Fig 43&44). These shoots proliferated further (Fig. 45) and increased in number (Fig. 46).

Rooting:

Efforts are underway to induce rooting at the cut ends of the regenerated shoots to obtain complete plantlets so that they could be transferred to the natural field conditions after acclimatization.

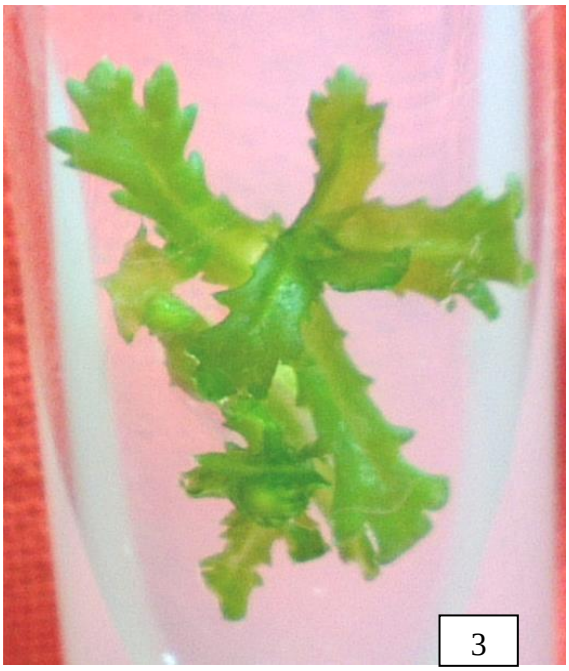
Axillary Bud Culture

Figure 3: Nodal bud break reported on MS + NAA (2.685 µM) + Kn (4.65 µM) after 12 days of inoculation.

Figure 4: Regeneration of shoots (4 – 5) from nodal explant on MS + NAA (2.685 µM) + Kn (4.65 µM) after 21 days.

Figure 5: Formation of 10 – 12 shoots after 4 weeks.

Figure 6: Formation of 20 – 22 shoots after 6 weeks.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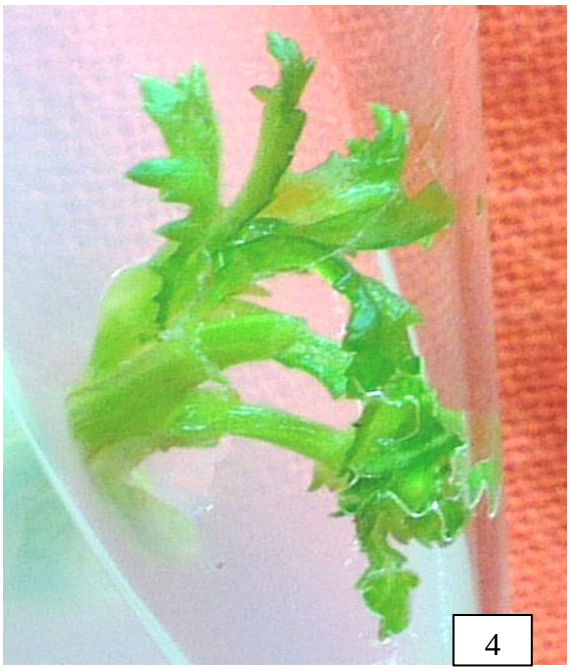
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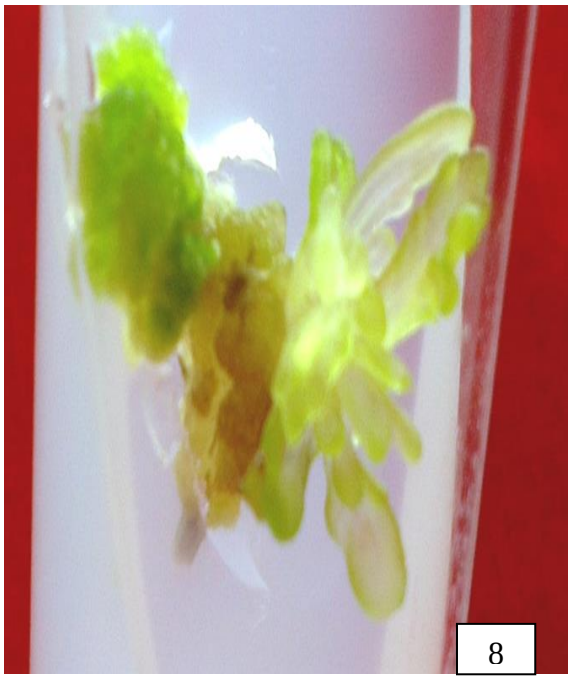
Figure 7: Nodal explant showing multiple shoot proliferation (10 - 12) on MS + NAA (5.37µM) + Kn (4.65 µM).

Figure 8: Nodal explant showing multiple shoot proliferation and callusing on MS + NAA (10.74µM) + Kn (4.65 µM).

Figure 9: Histogram depicting the effect of growth regulators on axillary shoot proliferation.

7



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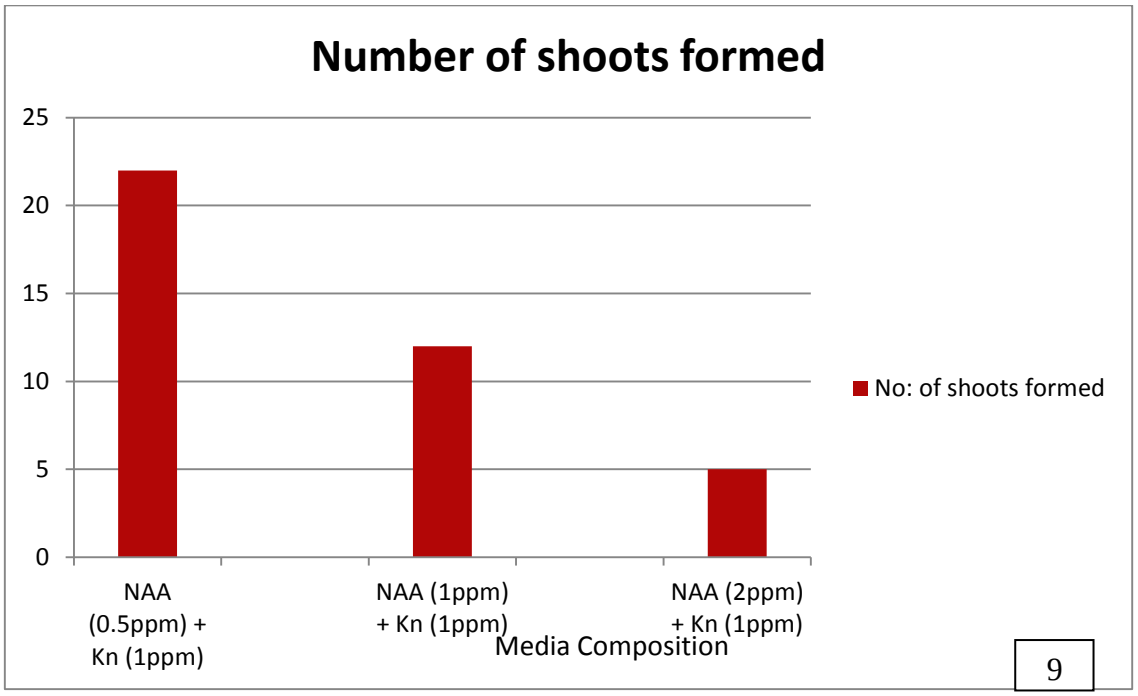
Number of shoots formed
25
20
15
10
5
0
NAA (0.5ppm) + Kn (1ppm)
NAA (1ppm) + Kn (1ppm)
NAA (2ppm) + Kn (1ppm)
Media Composition
No: of shoots formed

9

Leaf Culture

Figure 10: Initiation of root formation from leaf explant on MS + NAA (5.37µM).

Figure 11: Formation of 4 – 5 roots after 25 days of culture.

Figure 12: Elongation of roots after 5 weeks.

Figure 13: Formation of roots on MS + NAA (10.74 µM).

Figure 14: Formation of roots on MS + NAA (21.48 µM).


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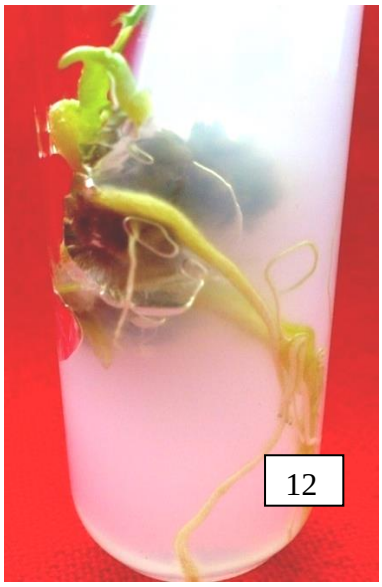
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Figure 15: Formation of roots on MS + IBA (4.9 µM).

Figure 16: Formation of roots on MS + IBA (9.8 µM).

Figure 17: Formation of roots on MS + IBA (19.6 µM).

15

16

17

Figure 18: Formation of roots on MS + IAA (5.71 µM).

Figure 19: Formation of roots on MS + IAA (11.42 µM).

Figure 20: Formation of roots on MS + IAA (22.84 µM).

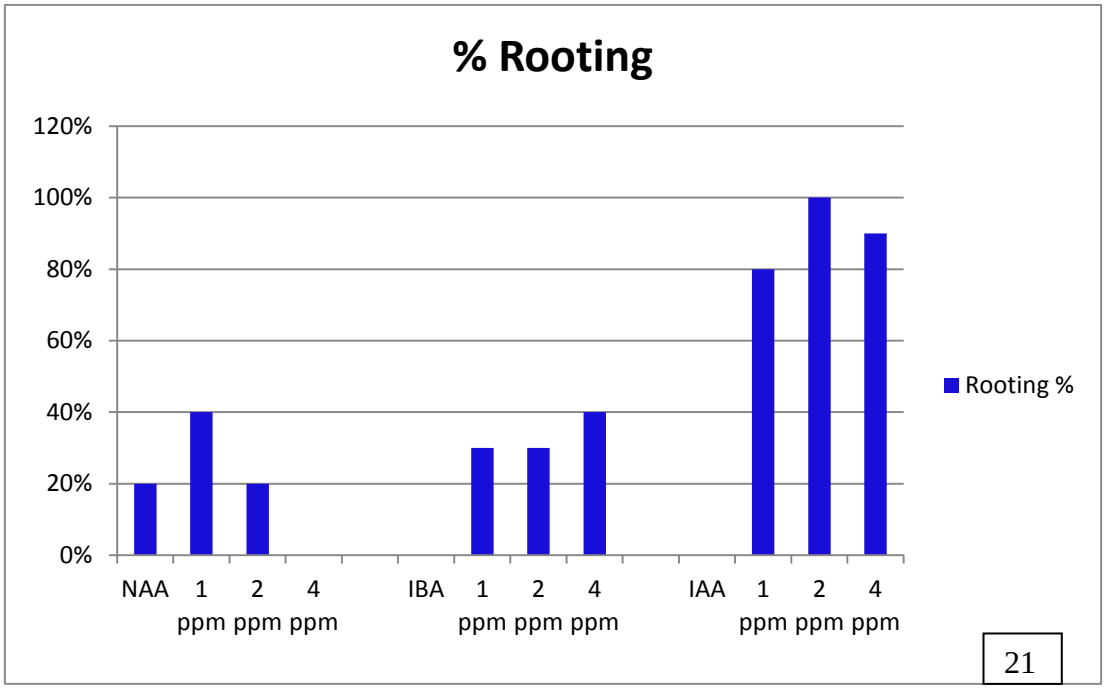
18

19

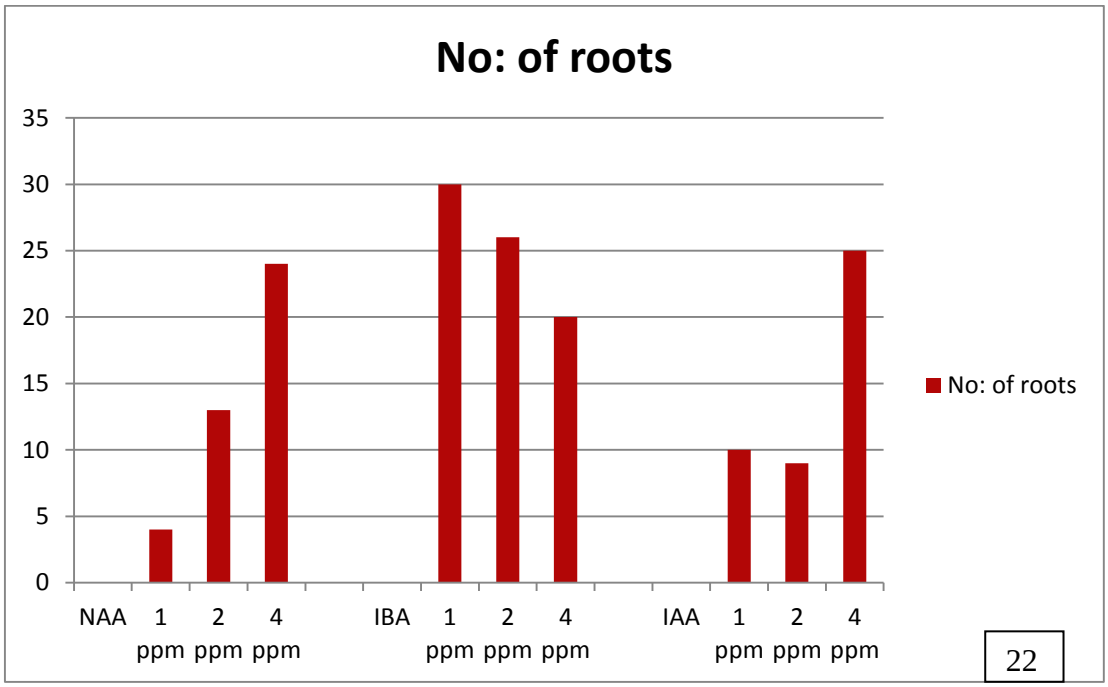
20

Figure 21: Histogram depicting %age rooting obtained from leaf explant on different auxins.

Figure 22: Histogram depicting the effect of growth regulators on root induction from leaf explant.


% Rooting
120%
100%
80%
60%
40%
20%
0%
NAA 1 ppm 2 ppm 4 ppm
IBA 1 ppm 2 ppm 4 ppm
IAA 1 ppm 2 ppm 4 ppm
Rooting %

21


No: of roots
35
30
25
20
15
10
5
0
NAA 1 ppm 2 ppm 4 ppm
IBA 1 ppm 2 ppm 4 ppm
IAA 1 ppm 2 ppm 4 ppm
No: of roots

22

Figure 23: Regeneration of shoots from the leaf explant on MS + NAA (5.37 µM) + Kn (4.65 µM) after 20 days of culture.

Figure 24: Formation of 12-15 shoots after 4 weeks.

23

24

Figure 25: Callusing along the entire surface of leaf explant on MS + 21.48 µM NAA + 4.65 µM Kn after 2 weeks of culturing.

Figure 26: Complete transformation of leaf explant into callus after 4 weeks of culture.

Figure 29: Differentiation of roots from callus formed on NAA (10.74 µM) + Kn (4.65 µM) after 6 weeks of culture.


25


26

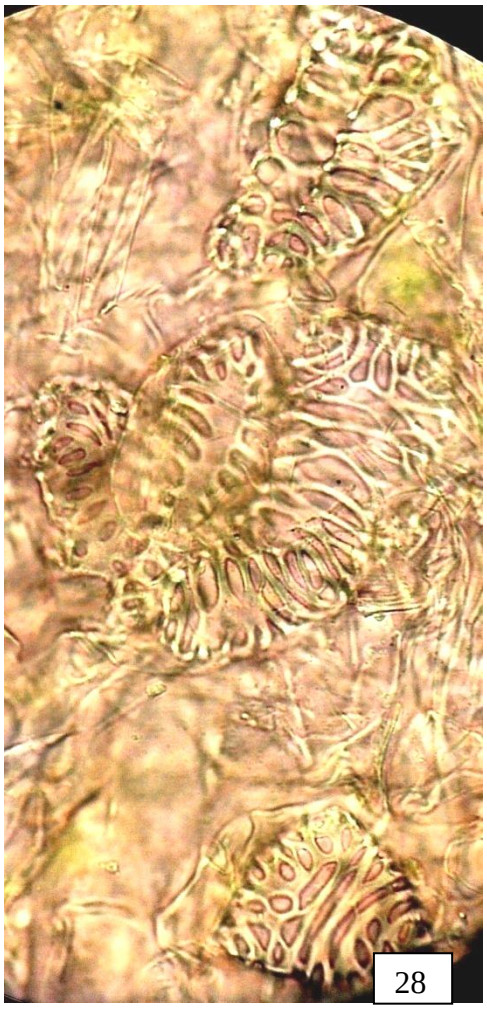

29

Microscopic study of Leaf Callus:

Figure 27: Highly elongated tracheids observed in four week old leaf callus.

Figure 28: Differently shaped tracheids observed in leaf callus with reticulate thickenings on their walls.

27



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Stem Culture

Figure 30: Direct root initiation from stem segment on MS + IAA (22.84µM).

Figure 31: Development of roots after 15 days of culture on MS + IAA (22.84 µM).

Figure 32: Browning of roots with passing time.

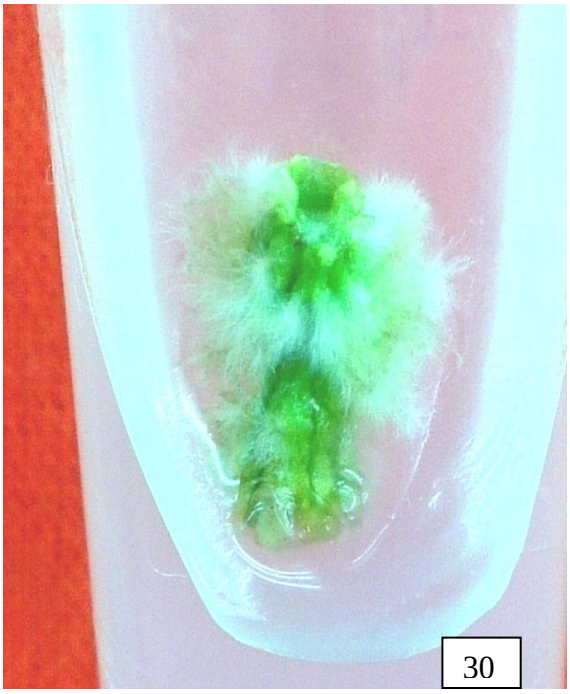
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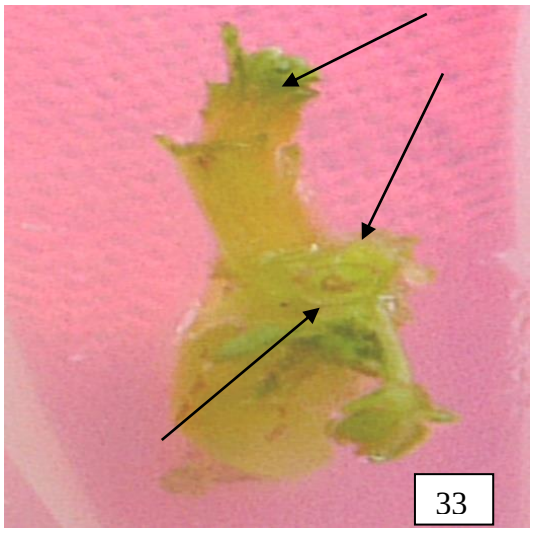
Figure 33: Shoot bud initiation from stem explant on MS + IBA (2.45 µM) + Kn (4.65 µM).

Figure 34: Formation of 8-10 shoots after 18-21 days.

Figure 35: Shoot bud initiation from stem on MS + IBA (4.9µM) + Kn (4.65 µM).

Figure 36: Formation of 7-8 shoots after 20 days.

Figure 37: Regeneration of adventitious shoots at one end of the stem segment along with callusing at the other end.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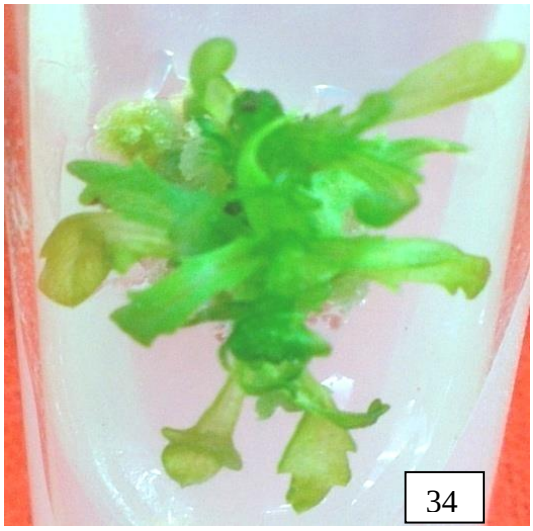
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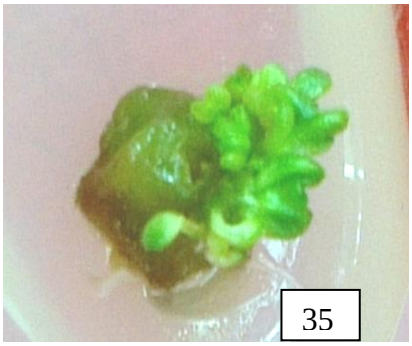
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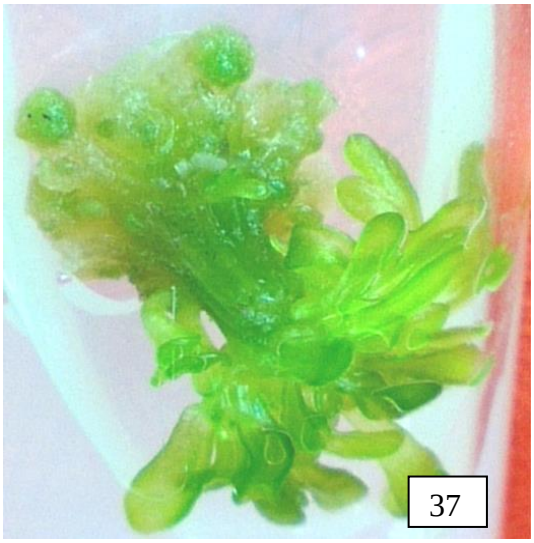
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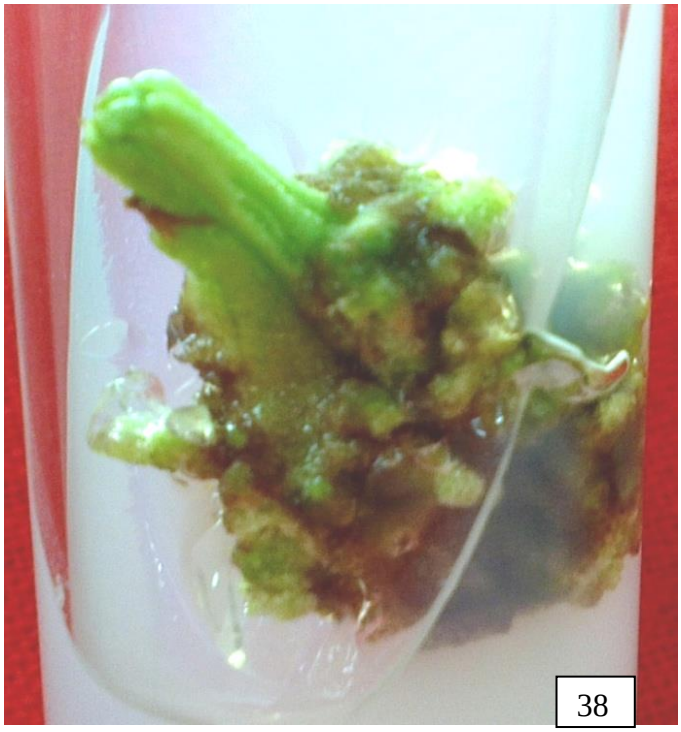
Figure 38: Callus induction from cut end of stem segment on MS + IBA (19.6 µM) + Kn (4.65 µM).

Figure 39: Complete conversion of stem segment into mass of compact callus after 5 weeks.



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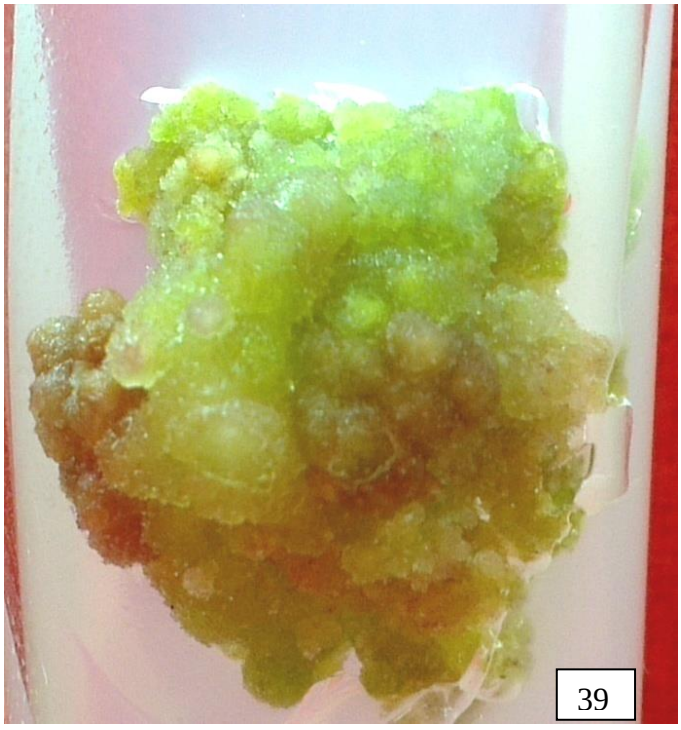

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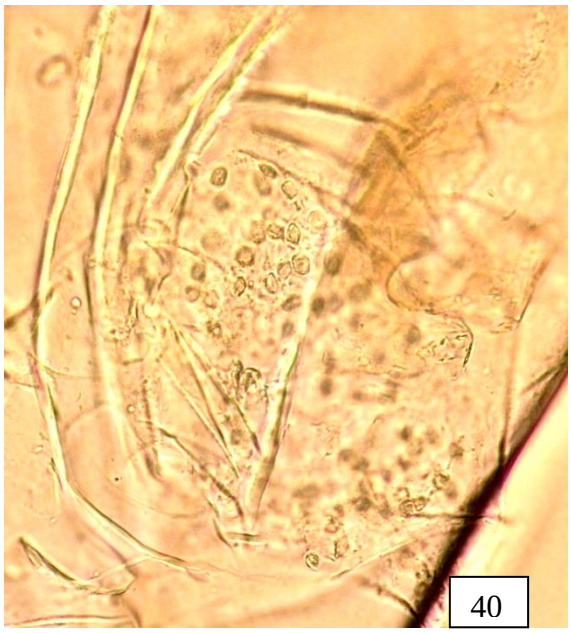
Microscopic study of Stem Callus:

Figure 40: Presence of numerous starch grains.

Figure 41: Compact arrangement of tracheids.

Figure 42: Magnified tracheids with reticulate thickenings on walls.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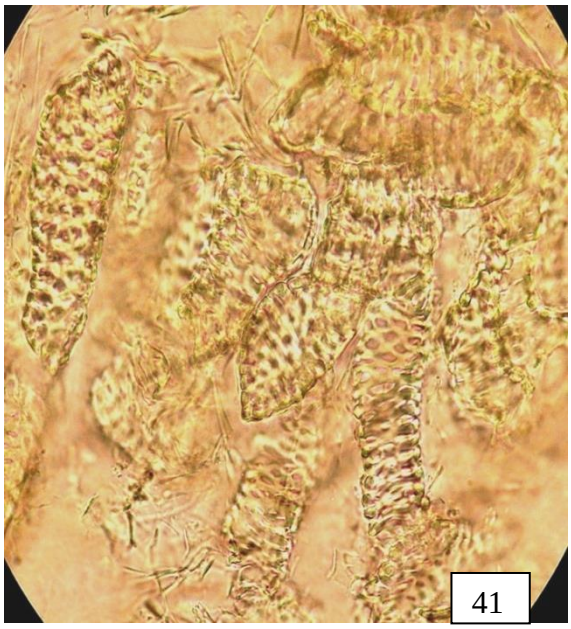
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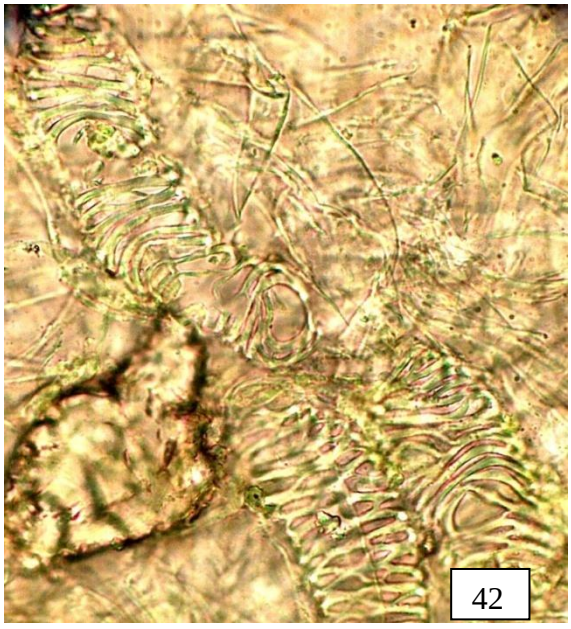
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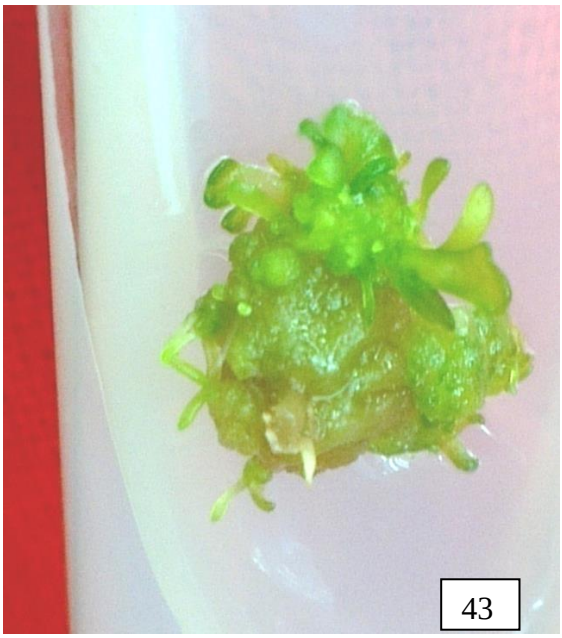
Figure 43: Regeneration of adventitious shoots indirectly from callus on MS + IBA (2.45 µM) + Kn (4.65µM).

Figure 44: Regeneration of adventitious shoots indirectly from callus on MS + IBA (4.9 µM) + Kn (4.65 µM).

Figure 45: Proliferation of regenerated shoots on MS + IBA (2.45µM) + Kn (4.65 µM).

Figure 46: Proliferation of regenerated shoots on MS + IBA (4.9 µM) + Kn (4.65 µM).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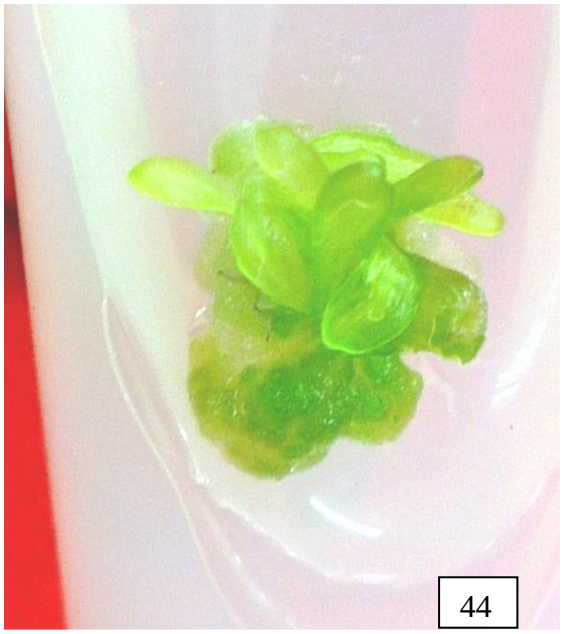
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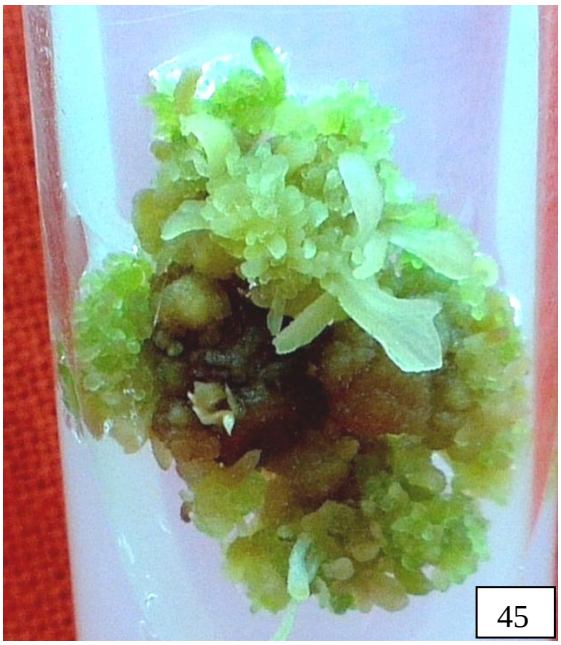
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DISCUSSION

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

Different explants excised from a healthy, mature field grown plant of *Chrysanthemum paludosum* like axillary buds, leaf segments and stem segments showed high efficiency of adventitious shoot formation.

Micropropagation through enhanced axillary branching is a reliable technique for clonal propagation as it ensures to maintain the clonal fidelity of plantlets regenerated and prevents somaclonal variations usually encountered in the plantlets regenerated indirectly from the callus. Type and concentration of growth regulators as well as the different combinations in which they are used, significantly effects the axillary shoot proliferation from the cultured explants. Cytokinins form a major class of growth regulators and are considered as a major factor affecting shoot proliferation from the explant. A wide range of cytokinins like Kinetin, BAP, Zeatin have been employed in shoot proliferation (Viji et al., 2010). Cytokinins in combination with lower concentrations of auxins have also been reported to effectively induce shoot proliferation in different explants used.

In the present investigation, axillary shoot multiplication was effected from the axillary buds inoculated on MS medium supplemented with various growth adjuvants in different combinations. Out of all the combinations tried, synergistic action of NAA (2.68µM) with Kn (4.65 µM) was most pronounced in forming numerous healthy, green shoots (20-22 in no.) after 6 weeks of culturing.

Waseem *et al*. (2009) reported maximum shoot initiation of *Chrysanthemum morifolium* from shoot tip explant on MS medium supplemented with BAP (1.0 mg/l) and BAP (1.0 mg/l) in conjunction with IAA (0.1 mg/l). Nalini (2012) reported MS medium with Kn 3.0 mg/1 + IAA 2.0 mg/l to be the best combination for regeneration of multiple shoots from shoot tips of

Chrysanthemum morifolium. Hedge *et al*. (2011) reported maximum shoot proliferation of *Rosa* using shoot tip and nodal explants on MS + 2 mg/l BAP + 0.2 mg/ l Kinetin + 25 mg/l adenine sulphate with maximum number of 25-30 shoots per explant. Maurya *et al*. (2013) reported 100 % shoot proliferation from nodal segments of *Rosa hybrida* on MS medium containing 2.0 mg/l BAP and 0.1 mg/l NAA.

In vitro micropropagation by *de novo* adventitious shoot formation is also a successful method for propagation of many ornamental plants. New adventitious shoots can develop directly from the explants like root, stem, petiole, leaf and floral parts or indirectly from the calli obtained from these explants. Choice of explant and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoots.

The plant material used in the present study exhibited high efficiency of *de novo* adventitious root and shoot formation directly from the stem and leaf segments. MS basal medium supplemented with NAA (1ppm) + Kn (1ppm) favoured direct adventitious shoot formation from the leaf explants and 12-15 shoots were formed after 4 weeks of culture. For the stem segments, IBA (02.45-4.9 µM) + Kn (4.65 µM) was found to be best for the induction of adventitious shoots.

Lee *et al*. (1997) reported adventitious shoots regeneration from leaf discs of *Chrysanthemum coronarium* on MS medium containing BA (2.5 mM) and NAA (2.5 mM). Song *et al*. (2011) reported a high frequency shoot induction (100%) from leaf, petal, petiole, and stem segments of *Chrysanthemum morifolium* on MS medium supplemented with 6.66 µM BA, 8.56 µM IAA and 0.46 µM Kn. Naing *et al*. (2014) reported adventitious shoot regeneration directly from leaf segments of the *Chrysanthemum* cv. Vivid Scarlet on MS + 1 mg/L of BA and 2 mg/L of NAA

Auxins play a major role in root induction through its effect on the first cell division which forms root initials (Farooq et al. 2008). In the present work, direct *de novo* adventitious root formation was observed both from leaf and stem segments of plant. High propensity of rooting was observed from leaf explants on MS medium augmented with NAA, IBA and IAA (1-4 ppm). For the auxins like NAA and IAA tried, a general observation made was that as the concentration of these auxins increased from 1 ppm onwards , the number, length and thickness of roots increased . However with IBA , an increase in the concentration resulted in the decrease in the number of roots formed.

IBA (1ppm) gave best results for rooting from leaf explants with maximum number of roots formed. On the other hand IAA was found to exhibit earliest and fastest induction of *de novo* adventitious root formation from leaf explants. The morphology of roots also varied with different concentrations of auxins used. In general, it was observed that at lower concentrations, roots were thin and slender and green in color and as concentration of auxins increased, roots became more thick and brownish in color. Roots formed on the surface of explant above medium bore dense root hairs whereas roots growing into the medium were invariably devoid of 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 and it was observed that the level and type of auxin required for dedifferentiation and optimal callusing varied among the the leaf and stem segments. Murashige and Skoog's agar gelled medium supplemented with NAA (4 ppm) + Kn (1 ppm) turned out to be optimal for initiation and sustained growth of calli from leaf explants. The calli obtained from leaf were green, soft and friable. For stem parts, MS + IBA (4 ppm) + Kn (1ppm), was found to be the optimal chemical milieu for best callus induction. The callus formed was yellowish green, very hard and compact and did not show sustained growth.

Both stem and leaf calli were heterogeneous i.e. showed high variability in cell shape and size. Cell shape ranged from spherical, ovoid and elliptical to aberrant shapes. Histogenetic differentiation in form of tracheids was observed in all calli. Tracheids occurred in groups and possessed reticulate thickenings on their walls. It was also observed that tracheids multiplied, increased in number and arranged into compact groups with advancement of time as callus proliferated. This observation is in support with the fact that cell division must precede the formation of vascular elements.

Organogenetic differentiation was also observed in both leaf and stem calli. Callus formed from leaf on NAA (10.74 μM) + Kn (4.65 μM) exhibited root differentiation with thin, long and slender roots. Caulogenic differentiation was observed from stem callus obtained on IBA (2.45-4.9 μM) + Kn (4.65 μM). Many clusters of shoots were formed on the surface of callus. It seems not improbable that different explants have certain biochemical environment within their cells in the intact plant, which became accentuated during callus growth resulting in preferential differentiation of roots, leaves and shoots from their respective calli.

Bhattacharya *et al.* (1990) reported indirect micropropagation of *Chrysanthemum morifolium* from leaf and stem callus on MS + 0.1 mg/L IAA and 0.2 mg/L BAP. Mandal *et al*. (2000) reported callusing of stem segments of *Chrysanthemum morifolium* and development of shoot buds in 50% calli on MS medium with 1.07 µM NAA + 9.29 µM Kn. Vantu (2005) reported callusing from stem segments of *Chrysanthemum morifolium* on MS medium with 2mg/l BAP and 0.2 mg/l NAA. These reports are in accordance with the findings of present work. Garcia *et al*. (2011), reported organogenesis from callus derived from stem and leaf segments of *Passiflora suberosa* with highest regeneration efficiency from stem segments cultured on MS medium containing 44.4 µM BA. Reddy and Bopaiah (2012), reported regeneration of plantlets from callus derived from leaf of *Anthurium scherzeriaum* on MS medium supplemented with BAP (1.0 mg/L) and NAA (0.5 mg/L).

This is the first report of *in vitro* morphogenetic studies on *Chrysanthemum paludosum*. It is concluded that an efficient and reproducible protocol has been established for the large scale production of shoots through different techniques of micropropagation. The results obtained suggested high organogenetic potential of this plant which needs further investigation to establish a complete protocol for the mass cloning of this important ornamental plant.

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