IN VITRO PLANTLET REGENERATION OF CHRYSANTHEMUM (*Chrysanthemum morifolium*)

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DHAKA-1207

JUNE, 2017

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A Thesis

Submitted to the Department of Biotechnology,

Sher-e-Bangla Agricultural University, Dhaka

In partial fulfillment of the requirements

for the degree

of

MASTER OF SCIENCE (MS)

IN

BIOTECHNOLOGY

SEMESTER: JANUARY - JUNE, 2017

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CERTIFICATE

*This is to certify that the thesis entitled "**IN VITRO** **PLANTLET REGENERATION OF CHRYSANTHEMUM** **(Chrysanthemum morifolium)**" submitted to the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biotechnology, embodies the result of a piece of bona fide research work carried out by **MD. SHAHIDUL ISLAM KHAN**,* **Reg. No.: 11-04486** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

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ACKNOWLEDGEMENTS

The author would like to express his deepest sense of gratitude, endless praises and thanks to the Almighty Allah Who enabled him through His kind blessings to complete this piece of research work and to submit the dissertation.

The author expresses his deep sense of gratitude and profound respect to his research supervisor and chairman, ***Prof. Dr. Md. Ekramul Hoque****, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, for his constant supervision, valuable suggestion, scholastic guidance, continuous inspiration, constructive comments and immense help in conducting the research work and preparation of the dissertation.*

The author expresses his sincere appreciation, respect and immense indebtedness to the respected members of co-supervisor, ***Fahima Khatun****, Assistant professor, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, for cordial suggestions, constructive criticisms and valuable advice during research work.*

The author expresses his heartfelt gratefulness to ***Homayra Huq****, Associate Professor, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, for her inspiration and encouragement.*

The author also expresses his heartfelt gratitude to the members of the family for their blessings, encouragement and constant inspiration throughout his academic career. Thanks to all of his nearest and friends for their help and good wishes all the time.

June, 2017 ***The Author***

SAU, Dhaka

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(*Chrysanthemum morifolium*)

ABSTRACT

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207 from the period of February 2016 to June 2017 for *in vitro* regeneration of chrysanthemum where nodal segments were used as explants. Five levels of BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) and IAA (0.5, 1.0, 1.5, 2.0 and 2.5mg/l) were used for shoot and root induction. 2,4-D (0.5, 1.0, 1.5 and 2.0 mg/l) was used in combination with 2.0 mg/l of BA for callus and shoot induction. The highest frequency of shoot (80.00%) in minimum 7.60 days and maximum number of shoot (2.20, 2.60 and 3.00) at 14, 21 and 28 DAI (days after induction) were recorded from BA 2.0 mg/l. Moreover, the highest length of shoot and number of leaves was noticed from the 3.0 mg/l and 2.0 mg/l of BA, respectively at 14, 21 and 28 DAI. The highest frequency of callus (76.00%) was observed within 14.20 days in MS medium and weight of callus (1.80 g, 2.70 g and 3.50 g) was obtained with BA 2.0 mg/l+ 2,4-D 1.0 mg/l. The maximum number of shoot (3.20) at 40 DAI was obtained with BA 2.0 mg/l+ 2,4-D 1.0 mg/l within minimum 9.20 days. IAA 0.5 mg/l produced the highest percentage of root (80.00%) within 13.00 days while longer days required in other treatments. The maximum number of root (2.60, 3.60 and 5.40) was obtained from 1.5 mg/l IAA at 14, 21 and 28 DAI, respectively. The maximum length of shoot (2.48 cm, 2.84 cm and 3.66 cm) at 14, 21 and 28 DAI was noticed from the BA 2.0 mg/l+ IAA 1.0 mg/l. In combined effect the highest percentage (76%) of root induction was recorded with BA 3.0 mg/l+ IAA 1.5 mg/l in minimum 12.20 days and BA 2.0 mg/l+ IAA 1.0 mg/l gave the highest number of root 2.40, 3.20 and 4.20 at 14, 21 and 28 DAI, respectively. The survival rate was 80% in shade condition which was 75% in open atmospheric condition. Therefore, an efficient protocol has been developed for *in vitro* regeneration of chrysanthemum which has great commercial value for year round production in Bangladesh.

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ABBREVIATIONS AND ACRONYMS

Agril.	:	Agriculture
Biol.	:	Biological
cm	:	Centimeter
CRD	:	Completely Randomized Design
DMRT	:	Duncan's Multiple Range Test
Conc.	:	Concentration
DAI	:	Days After Inoculation
et al.	:	And others (at elli)
FAO	:	Food and Agricultural Organization
IASC	:	International Aloe Science Council
g/L	:	Gram per litre
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
NAA	:	*a*- Napthalene acetic acid
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
MS	:	Murashige and Skoog
Int.	:	International
2-ip	:	2-isopentenyladenine
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
TDZ	:	Thidiazuron
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Etcetera

CHAPTER I
INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium)* commonly known as Gul-e-Daudi or Autumn Queen belongs to the family Compositeae (Asteraceae) (Arora, 1990). It is highly attractive and charming short day plant, which behaves both as an annual as well as perennial flowering herb. The plant height ranges from 1-3 feet. The leaves are alternate and toothed, roots are adventitious and the stem is woody solid. The flowers bloom in early winter with a wide range of color, shape and sizes. The flower color ranges from white and cream through the shades of yellow, pink, bronze, red, deep purple and green (Arora, 1990).

The common commercially available chrysanthemum is *Chrysanthemum morifolium* Ramat. It is native to the northern hemisphere, chiefly Europe and Asia. In the southern parts of the country, it is mostly grown in farmer's fields for supplying loose flowers to the market for garlands, hair decoration by the ladies and for offering to God. While yellow colored flowers are preferred in the south, in the north, various hues of red, purple, yellow and white are found to be grown in abundance.

Chrysanthemum accounts for 35 percent of the total cut flower production. Chrysanthemum is the world's second most economically important floricultural crop following the rose (Teixeira da silva, 2003). Chrysanthemum is one of the largest cut-flower among the ornamental plants traded in the global flower market. Chrysanthemums are important for their outstanding aesthetic beauty. They can be propagated vegetatively either through root suckers or terminal cuttings; this conventional process of shoot cutting is very slow (Nhut *et al*., 2005). This approach, however, is inadequate to attain fast multiplication rate, as these conventional propagating methods are very slow, time consuming and tiring. Secondly, cuttings obtained repeatedly from mother plants may be subjected to any virus infection and degeneration, thereby increasing production costs (Hahn *et al.*, 1998).

High frequency regeneration of plants from the *in vitro* cultured tissue is a pre requisite for successful application of tissue culture techniques for crop improvement (Akter, 2001). Chrysanthemum *in vitro* culture was extremely useful for producing a huge number explants in a short time as stated by Dao *et al.* (2006). Tissue culture studies in chrysanthemum are being done as a tool for mutation induction and as a means of micropropagation. However, the ability to regenerate plants from a single cell of florets is a useful approach to establish a mutant in pure form and facilitate the production of a wide range of new flower cultivars as stated by Mandal *et al.* (2000).

The commercial cultivars are usually propagated vegetatively through terminal cuttings and root suckers . These conventional propagation methods not only take longer time to flower, but also useful for large scale production. Regeneration through *in vitro* culture has become now a viable alternative to the conventional propagation methods. However, clonal propagation through *in vitro* culture can enhance the multiplication rates (Sauvaire and Galgy, 1978).

In tissue culture, the use of plant growth regulators play a pivotal role in influencing different plant processes comprising mostly of growth, differentiation and development for example, culture establishment, shoot initiation, callogenesis, embryogenesis, rooting, etc (Hobbie, 1998). Pierik (1987) stated that cytokinins are often used to stimulate growth and development, Kin and benzylaminopurine (BAP) being in common use. Although the presence of a cytokinin is almost always advantageous, and required, optimum rates of shoot initiation generally occur with the combinations of auxins and cytokinin (George, 1993).

Many researchers developed micropropagation techniques for Chrysanthemum using different parts such as shoot tip, stem and apical meristem but very few reports are evident on using nodal segment as explants for chrysanthemum regeneration. Present study is designed using nodal explants which may be fruitful to cultivate the plant in our soil and environment.

So due to high popularity and demand for chrysanthemum in the world as well as Bangladesh it has become one of the first commercial targets for micropropagation.

Hence, tissue culture technique can be utilized for its large scale production. Therefore, the research program is designed with the following objectives-

Objectives:

i. To Study the role of phytohormone on *in vitro* regeneration in Chrysanthemum.
ii. Assessment of best hormonal combination for *in vitro* regeneration of Chrysanthemum.
iii. Establishment of *in vitro* regeneration protocol of Chrysanthemum.

CHAPTER II
REVIEW OF LITERATURE

The present investigation involved *in vitro* micropropagation of chrysanthemum, plant regeneration from callus culture of nodal segment explants followed by genetic stability of plantlets. The related reviews have been cited under the following sections:

In vitro Propagation of Chrysanthemum

Chrysanthemum is commonly known as Autumn Queen. It belongs to the family *Compositeae* (Asteraceae). It is highly attractive and charming short day plant, which behaves both as an annual as well as perennial flowering herb. Regeneration of Chrysanthemum plantlets through *in vitro* culture was obtained by using nodal segment explants. These explants were treated with different concentrations of auxins (IAA, IBA) and cytokinins (BAP) alone and in combinations. Nodal segments of Chrysanthemum were placed on MS medium supplemented with different concentrations of BAP with IAA. The highest results were observed on MS medium containing 1.0 mg/l BAP + 0.1 mg/l IAA, showed 90% shoot initiation and 5.5 ± 0.51 average length of shoot per explant. Nodal segments of Chrysanthemum were cultured in MS media with different concentration of BAP. The highest results were observed on MS medium containing 1.0 mg/l BAP, shown 93% shoot proliferation, 5.7 ± 0.22 cm average lengths of shoot per explant and 4.4±0.88 nodes per explant. The regenerated shootlets were rooted on MS medium and ½ MS medium with different concentration of IBA. The highest results were observed on ½ MS medium containing 0.2 mg/l IBA showing 90% rooted micro cuttings. 9 ± 0.19 average length of root was observed per explant and 11.8 ± 0.75 numbers per explant of Chrysanthemum (Labade *et al.*, 2016).

Chakrabarty *et al.* (2000) regenerated shoots from basal part of ray florets of *Chrysanthemum morifolium* cv. Colchi Bahar on MS medium supplemented with 0.2 mg/l NAA and 1 mg/l BAP.

Callus induction and shoot regeneration were studied in Chrysanthemum cultivars “Zipri” and “Shyamal Dark Pink” by Wankhede *et al.* (2000). For callus induction leaf bits and stem discs were cultured on MS medium supplemented with 2, 4-D (1.0, 2.0,

3.0, or 4 mg/1), BAP (0.l or 0.2 mg/l) + NAA (0.l or 0.2 mg/1), or BAP (0.50, 1.25, or 1.50 mg/l). Callus induction and growth were most pronounced in "Zipri' grown in MS medium containing either l mg/1 2,4-D, 0.2 mg/1 BAP + 0.2 mg/l NAA or 0.5 mg/1 BAP. In "Shyamal Dark Pink" the induction and growth of callus was maximum in the MS medium containing 2 mg/l 2,4-D, 0.1 mg/1 BAP + 0.1 mg/l NAA, or 0.25 mg/l BAP. In both the cultivars, leaf explant produced more callus than stem explants. Shoot formation was studied using shoot tip and axillary bud explants cultured in MS medium with BAP (0.50, 0.75, 1.00 or 1.25) mg/1 + IAA (0.5, l.0, 1.5 or 2.0) mg/l or kinetin (0.5 or 1.0) mg/1+ IAA (0.5 or 1.0) mg/l. In "Zipri" multiple shoot formation was highest in shoot tip explants cultured on the MS medium containing 1.25 mg/1 BAP + 1.0 mg/l IAA. In "Shyama Dark Pink" the maximum shoot formation was observed in shoot tip explants grown in the MS medium supplemented with 1.0 mg/l BAP + 2.0 mg/l IAA.

Meena *et al.* (2001) conducted *in vitro* plant regeneration from callus cultures of chrysanthemum. Explants, young leaves, nodes and internodes of two chrysanthemum cultivars namely "Snow Ball" and "Miss Universe" were cultured on MS basal medium supplemented with various concentrations (0.5-2.0 mg/l) of growth regulators (IAA, NASA, 2,4-D, BAP, BA and kinetin) alone and in various concentration and combinations. Regenerations were best in nodal explant followed by young leaves and internodes. Plantlets raised from the callus of the nodes were successfully transferred to pots and later to soil.

Seo *et al.* (2003) cultured leaf explants of chrysanthemum (*Dendranthema grandiflora*) on MS medium supplemented with various concentrations of growth regulators. The highest frequency of shoot formation was observed on MS medium supplemented with 1 mg/ 1 BA + 0.3 mg /1 2,4-D in cultivar "Puma" and with 2 mg/1 NAA + 0.5 mg /1 BA in cultivar "Subangryeok".

Chung and Park (2005) developed protocol for shoot regeneration with leaf disk explants of garland chrysanthemum (*Chrysanthemum coronarium* L.). The optimal concentrations of NAA and BA used were 0.2 mg/1 and 0.5 mg/1, respectively.

Chitra *et al*. (2006) reported clonal propagation of chrysanthemum (*Dendranthema grandiflora*). The frequency of multiple shoot regeneration response was 95% and 80% for nodal segments and shoot tips cultured on the media containing MS + 2.0 mg/1 + 1.0 mg/1 BAP + 1.0 mg/1 IBA. *In vitro* raised plantlets were successfully transferred to potting media and were established in main field.

Jeremovic *et al.* (2006) cultured shoots of chrysanthemum cv. "White Spider" on MS medium supplemented with BAP and NAA (1.0, 0.1 mg/1, respectively). Rooting of shoots (100%) was done on MS medium without hormones and it was very successful after ten, two or eight years of micropropagation. The condition of growing chrysanthemum plants at greenhouse was excellent and at appropriate photoperiod "*in vitro*" plants flowered 90.3%.

Trifuncovic *et al.* (2006) achieved morphogenesis in stem segment callus cultures of chrysanthemum cultivars Regan Sunny (RS) and White Spider (WS) on MS medium supplemented with 0.5 mg /l NAA and 1 mg/1 BAP.

Liu *et al.* (2007) induced large number of buds directly from epicotyl and hypocotyl explants of *Chrysanthemum cinerariifolium* on MS medium supplemented with 0.3 mg/l NAA. Root induction and development in shoots was achieved within 15 days of inoculation on half strength MS medium supplemented with 0.2 mg/l and 0.1 mg/ l of IAA.

Ilahi *et al.* (2007) cultured nodal explants of chrysanthemum on MS medium containing different combinations of growth hormones. A reasonable callus was formed on explants cultured on MS medium containing 0.5 myl each of BAP and NAA after 3 weeks. The callus then exhibited various embryonic developmental stages and gave rise to normal seedlings. The seedlings were acclimatized and transferred to natural conditions. The plants ultimately flowered exhibiting superior quality and early maturity compared to stock plants.

Minas and Trigiano (2007) mass propagated chrysanthemum by culturing apical meristems in MS medium supplemented with 4.5 mg/1 IBA + 0.009 mg/1 IBA + 55.7 mg/1 ascorbic acid.

Nahid *et al*. (2007) developed a method to initiate multiple shoots from petal explants of *Chrysanthemum morifolium*. Petal explants (7mm) were cultured on Murashige and Skoog's medium containing different concentration and combinations of plant growth regulator (cytokinin or auxin and cytolcinin) for callus formation and shoot induction. Highest number of shoots were obtained in MS media supplemented with 2.0 mg /1 IBA and 0.1 mg/1 kinetin and 0.1 mg/1 NAA, respectively.

Datta *et al.* (2008) reported direct shoot organogenesis from original and gamma radiated ray florets of chrysanthemum (*Dendranthema grandiflomm*cv. Puja) within two weeks of culture initiation on MS medium supplemented with 1.0 mg/1 BAP + 0.5 mg/1 NAA.

Jaramillo *et al.* (2008) induced organogenesis in three varieties of chrysanthemum (White Albatross and Escapade) on MS medium supplemented with 4.83 pM BAP and 4.83 pM NAA + 13.32 pM BAP.

Lokanaha and Maheshvwaramma (2009) developed a protocol for *in vitro* regeneration from callus obtained from explants viz. shoot tip, leaf, auxillary bud and internodal segment of Chrysanthemum c.v. "SNOW CEM" cultured on MS medium supplemented with various combination of auxins (IAA and NAA) and cytokinins (BAP and kinetin). Highest regeneration frequency (45.3%) of "SNOW CEM" was obtained with axillary bud explant on MS medium supplemented with IAA (1.5 mg/1) and BAP (2.5 mg/1). MS media supplemented with BAP (1.0 and 2.0 mg/1) along with the lower concentrations of IAA (0.1 and 0.2 mg/1) showed better results as compared to other concentrations and combinations. Satisfactory rooting was obtained in half strength MS media supplemented with indole butyric acid (0.2 mg/l).

Zalewska *et al.* (2010) cultured shoots of five chrysanthemum cultivars on MS media, each shoot divided into three equal Zones: distal, central and proximal. Two single - node explants were isolated from each Zone and cultured on MS medium without any

added growth regulators. After 10 weeks of culture, 50 per cent of the shoots developed from axillary buds. The remaining shoots were sub-cultured on rooting medium containing IBA (0.2 mg/l).

Murgayanti *et al.* (2010) cultured leaf explants of chrysanthemum on MS medium supplemented with 3% sucrose, 0.8% agar and combination of various growth regulators. The first factor was three different concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D) (0.0, 1.0 and 2.0 mg/1) and the second factor was four different concentrations of kinetin (0.0, 0.1, 0.5 and 1.0 mg/l). The callus regenerated on MS medium containing l mg/l BAP.

Fu Yun-liu (2010) established rapid propagation system to provide the basis for scale production of Chrysanthemum nankingense. Top buds and axillary buds of chrysanthemum cv. nankingense H.M. were used as explants for rapid propagation. The results showed young bud induction after 5 days of culture on half strength MS medium supplemented with BA 0.5 mg/l and NAA 0.1 mg/l. The medium for rooting was half strength MS medium with NAA 0.1 mg/l. The rooting ratio was 100%. They reported that the culture medium with different hormone combinations for culturing of chrysanthemum cv. nankingense H.M. should be chosen according to the developmental stages.

Waseem *et al.* (2011) developed efficient plant regeneration system from the nodal segments of *Chrysanthemum morifulium* L. Nodal segments, after sterilization with 1.0 % mercuric chloride for three minutes were inoculated in Murashige and Skoog (MS) media with varied concentrations of indole acetic acid (IAA), benzylaminopurine (BAP) and their combinations. Different parameters including shoot initiation percentage, average number of shoots per explant, length of shoots (cm), number of leaves per shoot and number of nodes per shoot were studied during the course of study. Intermediate level (0.3 mg/1) of IAA exceeded all the other concentrations of IAA by producing 80.0 % shoot initiation, an average of 4.0 shoots per explants, 5.1 cm long shoots, 11.3 leaves and 5.6 nodes per shoot. Similarly, intermediate level of BAP (1.0 mg/1) showed its supremacy over all the other concentrations used as it produced 100 % shoot initiation, 4.9 shoots per explant, 5.8 cm long shoots, 13.4 leaves and 6.3 nodes per shoot. When

the combination of different concentrations of IAA and BAP were used, significant results regarding the regeneration of chrysanthemum plantlets were also achieved. MS media supplemented with lower concentrations of IAA (0.1 and 0.2 mg/1) along with intermediate levels of BAP (1.0 and 2.0 mg/1) had a favorable effect on the regeneration of chrysanthemum plantlets using nodal segments of chrysanthemum.

Mani and Senthil (2011) reported *in vitro* propagation of chrysanthemum to have potential for fast multiplication of superior genotypes, allowing the exploitation of maximum genetic gain achieved in the breeding program. Callus induction from leaf explant in MS medium containing 1.5 mg/1 2, 4-D was found to be 100 % from petal explant it was found to be 100 % in MS medium containing 2.0 mg/1 2, 4-D. The best friable calli were subjected to suspension culture in MS media supplemented with 1.0 mg/1 BAP for somatic embryos. All calli in suspension gave rise to somatic embryos, which were regenerated in MS media supplemented with various concentration of BAP. The regenerated plantlets were elongated on MS media supplemented with 0.1 mg/1 BAP + 2.0 mg/l kinetin and rooted on MS basal medium containing IBA (0.1 mg/1).

Keresa *et al.* (2012) reported axillary bud shoot proliferation and somatic embryogenesis in *Dendranthemx grandiflnra* (Ramat.) Kitamura cv. Palisade White on modified MS medium supplemented with 1 mg/1 naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/1 BA, 200 mg/1casein hydrosylate (CH) and 290 mg/1 proline. Proliferation rate of 3.2 microshoots axillary bud was obtained in MS medium supplemented with BA (0.1 mg/1) was used in combination with GA_3 (0.5 mg/1). The number of roots per shoot was higher using IBA (0.5 mg/1), but IAA (2 mg/1) promoted longer roots. Leaf explants were most responsive; demonstrating the highest percentage of embryogenesis (97.9%), followed by petiole and internode's stem explants (56.3 and 35.1%, respectively). The number of somatic embryos per embryogenic explant was highest on leaf explants. However, the best conversion rate (53.8%) of somatic embryos to plantlets was observed from petiole explants. For this reason, they reported petiole explants to be most suitable type for plant regeneration of chrysanthemum cv. Palisade White through somatic embryogenesis.

Verma (2012) reported root initiation in explants (axillary buds) of *Chrysanthemum morifoliums* on MS medium containing different concentrations and combinations of growth regulators. The best initiation with well differentiated micro shoots was achieved when the cultures were transferred to MS medium fortified with the various concentrations of auxins. Out of the different treatments tried 0.5 mg/1 NAA induced maximum rooting (88.66%) followed by control (79 %) as compared to 1.0 mg/1 NAA.

Kim and Naing (2013) induced somatic embryogenesis from *in vivo* grown leaf explants of Chrysanthemum cv. Euro incubated on Murashige and Skoog (MS) medium supplemented with 2.0 mg/l 2,4-dichlorophenoxyacetic acid and 2.0 mg/l kinetin, yielding the highest mean number of embryos (5.97) per explant after 5 weeks of culture. MS medium was observed to be the more effective in promoting the proliferation of somatic embryos than half-strength Murashige and Skoog medium.

Christian *et al.* (2013) cultured nodal explants from *in vitro* grown pyrethrum on Murashige an Skoog (MS) media supplemented with different concentrations of cytokininis, isopenyladenine (2iP), Benzylaminopurine (BAP), kinetin (KIN), Thidiazuron (TDZ), cysteine, 100 mg/1 Inositol, 2% sucrose. Rooting was evaluated using half strength MS media supplemented with Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA), 1-napthyleneaceic acid (NAA) and Dicholorophenoxy-acetic acid (2,4-D). Media without growth regulators was used as controls. Results showed that there were significant differences among cytokinins and auxins levels for the number and length of microshoots and roots, respectively. BAP at 40 pM gave the highest mean shoot number of 15.98 i 0.68 and the highest mean shoot length of 1.32 i 0.06 cm.

Soo (2014) used different concentrations and combinations of growth regulators to improve root organogenesis and micropropagation in *Chrysanthemum morifolium* (Ramat) cv. Hwiparam. Stem explants were cultured on 3 full strength basal MS (Murashige and Skoog, 1962), SH (Schenk and Hiberlandt, 1976) and B5 (Gamborg *et. al.*, 1972) medium. The best medium for root regeneration was investigated at 4 different concentrations (l/4, 1/z, 1 and 2).The best type of medium for root regeneration and growth was SH medium. The results showed, half strength of SH (1/ZSH) to be best for the number of root per explant (4.3) and root length (31.4 cm).

Kazeroonian *et al.* (2014) cultured petal explants of two chrysanthemum cultivars of “Resomee Splendid” on MS medium supplemented with different concentrations and combinations of plant growth regulators (PGRs). Results indicated the supremacy of “Resomee Splendid" over the other cultivar in regard to the shoot induction percentage with 62.22% vs. 49.63%. Maximum percentages of the shoot induction (93.33% vs. 73.33) were achieved in MS medium 3.0 mg/l benzylaminopurine (BAP) + 0.5 mg/l l-naphthaleneacetic acid(NAA) and 4.5 mg/l BAP + 1.0 mg/l NAA, while, in cultivar “Resomee Splendid” and “Reagan Elite Salmon” the highest number of shoots per explants (2.73 vs. 3.0) was obtained . Considering callogenesis, the later cultivar totally showed a greater response to the PGR treatments. Adventitious shoots were formed indirectly on the “Resomee Splendid” explants. While, considering the other cultivar, direct shoot formation was observed on the media fortified with either 3.0 mg/l BAP + 0.5 mg/l NAA or 4.5 mg/l BAP + 0.5 mg/l NAA. A combination of thidiazuron (TDZ) and NAA resulted in indirect shoot formation in both the cultivars. Regenerated shoots successfully elongated and formed roots on MS medium supplemented with IBA (2.0 mg/l) and were finally acclimatized and transplanted into soil. Treatments containing TDZ were totally inferior to the ones comprising of BAP in terms of both quantitative characteristics.

Tiller (2014) studied *in vitro* propagation technique to obtain new buds of kulo chrysanthemum. He aimed to discover the influence of NAA and BAP in kulo chrysanthemum bud multiplication involving completely randomized design which was arranged by factorial with treatments which consisted of 0 ppm, 0.5 ppm and 1 ppm of NAA combined with 1 ppm, 2 ppm, and 3 ppm of BAP. The explants used were kulo chrysanthemum nodules. Observed variables consisted of budding time, callusing time, number of buds, height of buds, number of leaves, number of roots, length of roots, percentage of grown cultures, percentage of unresponsive cultures, percentage of contaminated cultures. Data was analyzed using analysis of variance and continued by 5 % LSD test. The interactions between NAA and BAP and their influence on number of buds, height of buds, number of roots, root length were obtained. NAA and BAP individually influenced the number of leaves.

Zalewska and Tymoszuk (2014) reported mutation breeding in chrysanthemum. Ligulate florets of *Chrysanthemum grandiflorum* (Ramat) Kitam. 'Cool Time' were inoculated on the MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA. No significant effect of the inflorescence development stage (incompletely open with a partially visible disk or with the entire visible disk in which tubular florets do not produce pollen or completely open in which two or half of the whorls of tubular florets produce pollen) was obtained on the shoot regeneration efficiency. Most shoots regenerated on transversely or lengthwise-cut into half or on the entire pierced ligulate florets horizontally inoculated with the abaxial side on the medium.

Helmanto and Rivai (2015) studied formation of callus from various explants viz., leaf, hypocotyl, cotyledon, stem, zygotic embryo and other plant parts of chrysanthemum cultured in medium containing auxin compounds especially, 2,4-dichlorophenoxy acetic acid (2,4-D). The results showed MS medium containing 3 mg/l 2,4-D to be best treatment for inducing callus from leaf. However, MS medium containing l or 2 mg/l 2, 4-D were the best treatments for inducing callus from internode.

Kim *et al.* (2015) developed an efficient protocol for shoot regeneration from leaf explants of chrysanthemum (*Chrysanthemum morifolium* Ramat) cultivar Shinma. Maximum number of shoots per explant (9.7) was regenerated in leaf explants of 6-week-old donor plants cultured on Murashige and Skoog medium containing a combination of 0.5 mg/l 6-benzyladenine and 0.5 mg/l (1-naphthaleneacetic acid with initial dark treatment for 7 days. Among the different auxins tested, indole butyric acid 0.3 mg/1 was most effective for root induction and development. Ploidy levels were analyzed by flow cytometry. There was no ploidy variation between the regenerated plants and the mother plant grown under greenhouse conditions. The new protocol will facilitate genetic transformation and micropropagation of chrysanthemum cv. Shinma.

CHAPTER III
MATERIALS AND METHODS

3.1 Time and Location of the experiment

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of February 2016 to June 2017.

3.2 Experimental materials

3.2.1 Source of materials

The planting materials of *Chrysanthemum morifolium* were collected from Agargaon Nursery, Sher-e-Bangla Nagar, Dhaka-1207.

3.2.2 Plant materials

The nodal segments of *Chrysanthemum morifolium* were used as experimental materials in the present investigation.

Plate 1. Nodal segment used as explant in *Chrysanthemum morifolium* regeneration.

3.2.3 Instruments

Metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121°C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glass ware

The Borosil glassware was used for all the experiments. Oven dried (250°C) Erlesnmeyer flasks, culture bottles, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation**.** The glassware were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely. Finally they were rinsed with distilled water and sterilized in oven at 160-180°C for 3- 4 hours.

3.2.5 Culture media

The degree of success in any technology employing cell, tissue and organ culture is related to few major factors. A significant factor is the choice of nutritional components and growth regulators. MS (Murashige and Skoog, 1962) medium supplemented with different phytohormones as per treatments were used as culture medium for shoot induction, shoot multiplication and maintenance and regeneration of roots from multiplied shoots. Hormones were added separately to different media according to the requirements. And for that stock solutions of hormones were prepared ahead of media preparation and stored at 4°C temperature.

3.2.6 Plant growth regulators (PGRs)

PGRs or hormones were added separately in different media according to the requirements. Following stock solutions of hormones were prepared ahead of media preparation and stored at 4°C temperature.

1. BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/ l) for shoot induction.
2. BA 2.0 mg/l with 2,4-D (0.5, 1.0, 1.5, 2.0 mg/l), respectively for callus and shoot induction.
3. IAA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ l) for root formation.
4. BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/ l) combination with IAA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) doses, respectively for shoot and root formation.

3.3 Preparation of stock solutions

The first step in the preparation of the medium was the preparation of stock solutions. As different ingredients were required in different concentrations, separate stock solutions for macronutrients, micronutrients, vitamins, growth hormones etc, were used.

3.3.1 Stock solution "A" (Macronutrients)

Stock solution of macronutrients was prepared up to 10 times the concentration of the final medium in 1000 ml of distilled water (dw). Ten times the weight of the salts required per litre of the medium were weighed properly and dissolved by using a magnetic stirrer in about 750 ml of distilled water and then made up to 1000 ml by further addition of distilled water (dw). To make the solution free from all sorts of solid contaminants, it was filtered through Whatman no. I filter paper. Then it was poured into a plastic container, labeled with marker and stored in a refrigerator at 4°C for later use.

3.3.2 Stock solution "B" (Micronutrients)

The stock solution of micronutrients was made up to 100 times the final strength of necessary constituents of the medium in 1000 ml of distilled water (dw) as described for the stock solution of macronutrients. The stock solution was filtered, labeled and stored in a refrigerator at 4°C.

3.3.3 Stock solution "C" (Iron sources)

This was prepared at 100 times the final strength of Fe_2SO_4 and Na_2 -EDTA in 100 ml of distilled water and chelated by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 4°C.

3.3.4 Stock solution "D" (Vitamins)

Each of the desired ingredients except myo-inositol were taken at 10 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots and stored at 20°C.

3.4 The preparation of the stock solution of hormones

In order to prepare hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Cytokinins were dissolved in few drops of acidic solutions (1N HCl) and auxins were dissolved in few drops of basic solutions (1N NaOH).

Hormones (Solute)	Solvents used
BA	1 N NaOH
IAA	1 N NaOH
2,4-D	1 N NaOH

The stock solution of hormones was prepared by following a general procedure. Ten ml of 70% ethyl alcohol or 1 (N) NaOH solvent and 100 mg of solid hormone was placed in a small beaker and then dissolved with the addition of sterile distilled water using a measuring cylinder and the volume was made up to 100 ml. The prepared hormone solution was then labeled and stored at 4±1°C for use up to two month. Growth regulators were purchased from Sigma, USA.

3.5 Preparation of culture media from stock solutions

To prepare 1 litre of MS medium the following steps were followed:

1. About 400 ml distilled water was taken in a flask.
2. One hundred ml of macronutrients, 10 ml of micronutrients, 100 ml of irons and 10 ml of vitamins were taken from each of these stock solutions into a 2-litre beaker on a heater cum magnetic stirrer.
3. Thirty g of sucrose was added to this solution and gently agitated to dissolve completely.
4. Required volume of hormone solutions was directly added to the solutions in the beakers.
6. The solution was poured into a 1000 ml measuring cylinder and the volume was made up to 1000 ml with addition of distilled water.
7. pH of the medium was adjusted to 5.8 with a digital pH meter with the help of adding 0.1 N NaOH or 0.1 N HCl as necessary.
8. To solidify the medium, 8 gm (0.8%) of Difco-brand Bacto-Agar was added to

the solution and the whole mixture was gently heated (avoiding boiling) with continuous stirring till complete dissolution of the agar.

9. Required volume of hot medium was dispensed into culture vessels. After dispensing and proper cooling of the medium, the culture vessels were plugged with cork and non absorbent cotton and marked with different codes with the help of a glass marker to indicate specific hormonal combinations.

3.6 Sterilization

Fixed volume of medium was dispensed into 100 ml conical flask or bottle. The flask or bottle was plugged with aluminum foil and marked with different codes with the help of a glass marker to indicate specific hormonal supplement. The conical flasks were then autoclaved at 15 psi of pressure at 121°C for 20 minutes.

3.6.1 Sterilization of culture medium

The media contained in glass vials were autoclaved at 15 psi and 121°C for 20 minutes. After autoclaving, the culture media were allowed to cool under normal condition.

3.6.2 Sterilization of glass wares and instruments

Beakers, test-tubes, conical flasks, pipettes, metallic instruments, like forceps, scalpels, needles and spatula were sterilized in an autoclave at a temperature of 121°C for 40 minutes at 15 psi of pressure.

3.6.3 Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all over the floors and walls with detergent or Lysol (germicide) followed by wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals. Generally, switching on the laminar airflow cabinet and sterilized the cabinet by wiping the working surface with 70% ethyl alcohol and then UV light was on for 30 minutes so that the working area of the cabinet is sterilized. After in the cabinet was delayed for at least 5 minutes to ensure safe environment.

3.7 Precaution to ensure aseptic condition

The cabinet was usually started half an hour before use and wiped with 70% ethyl alcohol to reduce the chances of contamination. The instruments like forceps, scalpels, needles etc. were pre-sterilized by autoclaving and subsequent sterilization was done by dipping in 70% ethyl alcohol followed by flaming and cooling. Hands were also

sterilized by wiping with 70% ethyl alcohol. Aseptic conditions were followed during each and every operation to avoid the contamination of culture.

3.8 Explants preparation and culture

3.8.1 Preparation of explants

The nodal segment was the starting material. It was obtained from developing shoots of *Chrysanthemum* grown under field conditions and was brought to the culture room. The nodes were washed with water in a beaker. The nodal segments were then cut in an optimum size required for inoculation in culture vial.

3.8.2 Surface sterilization of explants

The nodal segment of 2 to 3 cm size was taken in a beaker. Surface sterilization of explants was done as follows:

i. The nodes were cut as small size (2 to 3 cm) and rinsed with water.

ii. The nodal segments were soaked with Tween-20 solution having 10% concentration for 5 min.

iii. Washing with distilled water was done for several times.

iv. The explants were sterilized with 70% ethanol for 1min.

v. Then the explants were sterilized with 0.2% $HgCl_2$ for 2 min.

vi. The explants were rinsed with sterilized distilled water for at least 4 times.

vii. The final size of explants were made 0.5-1.0 cm.

viii. Finally the explants were transferred to the MS media carefully.

3.9 Culture of explants

3.9.1 Inoculation of explants in culture vial

The isolated and surface sterilized nodal segment was collected carefully through maintaining aseptic condition inside the laminar air flow cabinet. The individual nodal segments were directly inoculated to each of the culture vial containing 25 ml of MS medium supplemented with different concentrations of hormones as per treatment.

Explants were transferred to large sterile glass petridish or glass plate with the help of sterile forceps under strict aseptic conditions. The surface sterilized explants were inoculated carefully following proper sterilization process within laminar airflow cabinet. The mouth of culture vial was flamed before and after positioning of the explant on the medium.

Plate 2. Inoculation of explant (nodal segment) in medium

3.9.2 Incubation

The culture vials were transferred to culture racks and allowed to grow in controlled environment. The temperature of the growth room was maintained within 25±1°C by an air conditioner and a 16 hour photo period was maintained along with light intensity of 3000 lux for proper growth and development of culture.

Plate 3. Incubation of the inoculated plant materials

3.9.3 Maintenance of proliferating shoots

Initial sub-culturing was done after 15-25 days when the explants had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoot were cut into small pieces so that each piece would contain about one shoot. Leaf and blackish or brownish basal tissues were removed. Each piece was inoculated into a similar fresh medium. It was practiced at the interval of 10-20 days.

3.9.4 Root induction of regenerated shoots

When the shoots grew about 3-4 cm in length with 4-5 well developed leaves, they were removed aseptically from the culture vials and were separated from each other and again cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction.

3.10 Treatments

Five sub-experiments were conducted to assess the effect of different concentrations of BA and IAA on shoot proliferation and subsequent rooting of the multiplied shoot and BA with 2,4-D for callus and shoot induction.

Sub-experiment 1. Effect of BA on *in vitro* shoot induction potentiality in Chrysanthemum

Five levels of BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) and control (0.0 mg/l) were used.

Sub-experiment 2.

Effect of BA and 2,4-D on callus and shoot induction potentiality in Chrysanthemum

In this sub-experiment, nodes of Chrysanthemum were used as sources material to investigate the effect of BA and 2,4-D for callus induction.

Treatments: BA 2.0 mg/l with 0.5, 1.0, 1.5 and 2.0 mg/l of 2,4-D and control (0.0 mg/l) were used. The experiments were arranged in Completely Randomized Design (CRD) with five replications.

Sub-experiment 3.

Effect of IAA on root induction potentiality in Chrysanthemum

Five levels of IAA (0.5, 1.0, 1.5, 2.0 and 2.5mg/l) and control (0.0 mg/l) were used.

Sub-experiment 4.

Combined effect of BA and IAA on shoot and root induction potentiality in Chrysanthemum

Treatments:

In this sub-experiment, 25 combinations of BA and IAA and one control treatment were practiced. The combinations were as follows:

T_0 = Control

T_1 = BA 1.0 mg/l+ IAA 0.5 mg/l

T_2 = BA 1.0 mg/l+ IAA 1.0 mg/l

T_3 = BA 1.0 mg/l+ IAA 1.5 mg/l

T_4 = BA 1.0 mg/l+ IAA 2.0 mg/l

T_5 = BA 1.0 mg/l+ IAA 2.5 mg/l

T_6 = BA 2.0 mg/l+ IAA 0.5 mg/l

T_7 = BA 2.0 mg/l+ IAA 1.0 mg/l

T_8 = BA 2.0 mg/l+ IAA 1.5 mg/l

T_9 = BA 2.0 mg/l+ IAA 2.0 mg/l

T_{10} =BA 2.0 mg/l+ IAA 2.5 mg/l

T_{11}= BA 3.0 mg/l+ IAA 0.5 mg/l

T_{12} = BA 3.0 mg/l+ IAA 1.0 mg/l

T_{13} = BA 3.0 mg/l+ IAA 1.5 mg/l

T_{14} = BA 3.0 mg/l+ IAA 2.0 mg/l

T_{15} = BA 3.0 mg/l+ IAA 2.5 mg/l

T_{16} = BA 4.0 mg/l+ IAA 0.5 mg/l

T_{17} = BA 4.0 mg/l+ IAA 1.0 mg/l

T_{18} = BA 4.0 mg/l+ IAA 1.5 mg/l

T_{19} = BA 4.0 mg/l+ IAA 2.0 mg/l

T_{20} = BA 4.0 mg/l+ IAA 2.5 mg/l

T_{21} = BA 5.0 mg/l+ IAA 0.5 mg/l

T_{22} = BA 5.0 mg/l+ IAA 1.0 mg/l

T_{23} = BA 5.0 mg/l+ IAA 1.5 mg/l

T_{24} = BA 5.0 mg/l+ IAA 2.0 mg/l

T_{25} = BA 5.0 mg/l+ IAA 2.5 mg/l

The experiments were arranged in Completely Randomized Design (CRD) with five replications. Each of replications consisted of five culture vials.

Sub-experiment 5.

***Ex vitro* acclimatization and establishment of plantlets on soil**

Tissue culture derived plantlets were acclimatized in growth chamber, shade house and open atmosphere to find out the survival percentage.

3.11 Data collection

The observations on development pattern of shoots and roots were made throughout the entire culture period. Five replicates (single shoot per culture bottle) were used per treatment. Data were recorded after 14, 21 and 28 days of induction on culture media in case of shoot and root proliferation. In case of callus formation, data was recorded at 20, 30 and 40 DAI (days after induction). The following observations were recorded in cases of callus, shoot and root formation under *in vitro* condition.

1. Days for callus induction
2. Percentage (%) of callus response
3. Wt. of callus (gm)
4. Days to shoot initiation from callus
5. Number of shoots from callus
6. Days to shoot induction
7. Percentage (%) of shoot response
8. Number of shoot
9. Length of shoot (cm)
10. Number of leaves/shoot
11. Days to root induction
12. Percentage (%) of root response
13. Number of roots per shoot
14. Length of root/shoot (cm)

3.11.1 Percentage of callus induction

Number of explants formed callus were recorded and the percentage of callus induction was calculated as

$$\textbf{Percent callus induction} = \frac{\text{Number of explants induced callus}}{\text{Number of explants inoculated}} \times 100$$

3.11.2 Weight of callus

The fresh weight of callus was measured in (gm) using analytical balance.

3.11.3 Percentage of shoot induction

The numbers of shoots were produced per explant were recorded and the percentage of shoot regeneration was calculated as-

$$\text{Percentage shoot induction} = \frac{\text{Number of explants induced shoots}}{\text{Number of explants inoculated}} \times 100$$

3.11.4 Days to shoot induction

Days to shoot induction were calculated by counting the days from explants inoculation to the first induction of shoots.

3.11.5 Number of shoot per explant

Number of shoot per explant was calculated by using the following formula,

$$\text{Number of shoot per explant} = \frac{\text{Number of shoots per explant}}{\text{Number of observation}}$$

3.11.6 Length of shoot calculation

Length of the shoot was measured in centimeter (cm) from the base to the top of the plantlet by measuring scale. It was recorded at 7 days interval.

3.11.7 Number of leaf

Numbers of leaves produced on the plantlet were counted and the mean was calculated.

3.11.8 Percent of explants showing root induction

The number of roots were per explants were recorded and the percentage of root regeneration was calculated as-

$$\text{Percent (\%) of root induction} = \frac{\text{Number of shoot induced root}}{\text{Number of shoot incubated}} \times 100$$

3.11.9 Days required for root initiation

Number of days required for initiation of root from the day of inoculation was recorded.

3.11.10 Number of roots/plantlet

Average number of roots/plantlet was calculated as the following formula -

$$\bar{x} = \frac{\sum xi}{n}$$

Where,

$\overline{X}$ = Mean no. of roots/plantlet

∑=Summation

xi=No. of roots in i th observation

n = No. of observation

3.11.11 Length of roots

Root length was determined in centimeter (cm) from the base to tip of the roots. Average length of the root was calculated by the following formula-

$$\bar{x} = \frac{\sum xi}{n}$$

Where,

$\overline{X}$ = Mean no. of roots/plantlet

∑=Summation

xi = length of roots in i th observation

n = No. of observation

3.11.12 Percentage of established plantlets

The percentages of established plantlets were calculated based on the number of plantlets placed in the plastic pots and the number of plants finally survived. The percentages of established plantlet were calculated by using the following formula:

Percentage of established plantlets = $\frac{\text{Number of established plantlets}}{\text{Total number of plantlets}}$ X 100

3.12 Acclimatization

Acclimatization or "hardening-off" is a process by which *in vitro* propagated plants were made to adapt to an *in vivo* environment.

Step-1: After 35 days of culture on rooting media, the plantlets were taken out from culture vial with the help of forceps with utmost care to prevent any damage to newly formed root and dipped in gentle warm water to remove any traces of solidified agar media for acclimatization. Plastic pots (6×6 cm) were kept ready filled with garden soil and compost in the proportion of 1:1 respectively. Immediately after removing solidified agar media from newly formed root, the plantlets were then transplanted in to the pots with special care.

Step-2: After planting, the plantlets were thoroughly watered and were kept at 25±2 °C with light intensity varied from 2000–3000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

Step-3: Then the plants were shifted to shade house with less humidity and indirect sunlight. The top of the pots were covered with transparent plastic sheet and grew at room temperature and 70% RH for 14 days with periodic irrigation (2 days interval).

Step-4: After 3 weeks, the plants were transferred to the soil following depoting and poting into different pot having bigger size. The plants were watered periodically and upper layer of the soil was mulched occasionally whenever necessary.

3.13 Statistical analysis of data

Data recorded for different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The means for all the treatments were calculated and analyses of variance of all the characters were performed. Experiment was conducted in growth room and arranged in Completely Randomized Design (CRD) with 5 replications. The significance of difference between the pair of means was evaluated at 1% level of significance by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

CHAPTER IV
RESULTS AND DISCUSSION

Different investigations were made on induction of calli and their regeneration ability. The results obtained from the experiment are described and discussed here and analyses of variance (ANOVA) presented in Appendix I-XII. Presentation of results has been made in three phases. The first phase involves shoot regeneration potentiality of explant; the second phase shows callus induction ability with regeneration potential of shoot and the third phase involves root initiation from regenerated shoots.

4.1 Sub-experiment 1. Effect of BA on shoot induction potentiality in Chrysanthemum

The result of the effect of different concentrations of BA has been presented under following headings with Figure (1.1-1.2) and Table (1-3).

4.1.1 Days to shoot initiation

The different concentrations of BA showed the significant variation on days to shoot induction. The maximum days (18.00) to shoot induction were recorded in control followed by 5.0 mg/l (15.00 days) and 4.0 mg/l (13.20 days). On the other hand, minimum (7.60 days) was required in 2.0 mg/l BA followed by 1.0 mg/l (9.60 days) and 3.0 mg/l (11.00 days) (Figure 1.1).

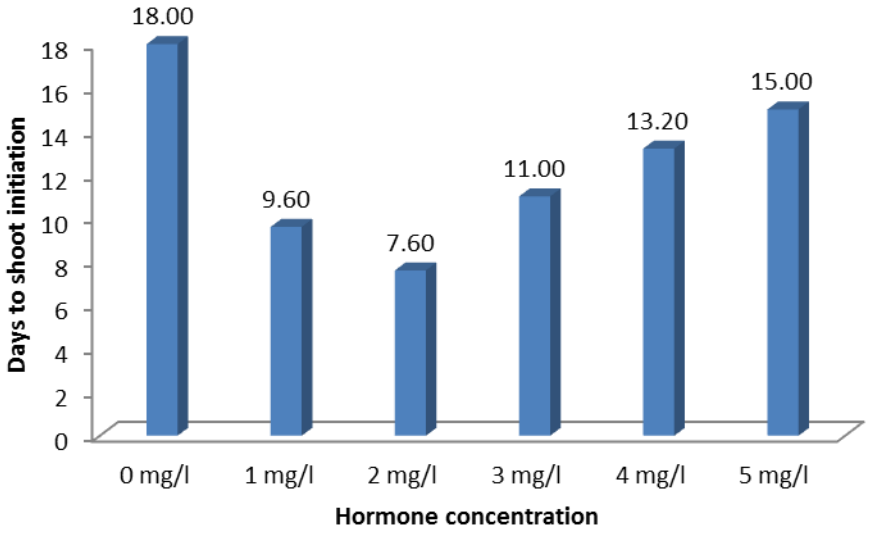

Figure 1.1 Effect of BA on days to shoot induction in Chrysanthemum

4.1.2 Percentage of shoot initiation

Significant variations were observed among different concentrations of BA on percentage of shoot induction. BA 2.0 mg/l had produced the highest frequency of shoot (80.00%), while the lowest percentage (40.00%) of shoot was produced in control treatment (Figure 1.2). Waseem *et al.* (2009) carried out shoot multiplication of Chrysanthemum from shoot tip explants in MS media mixed with 1.0 mg/l BAP that produced maximum shoot initiation percentage (93.3%). This result partially supported present findings. Those variation may be due to the age, nature, and the physiological state of the explants. (Bajaj, Y.P.S. *et al*., 1991).

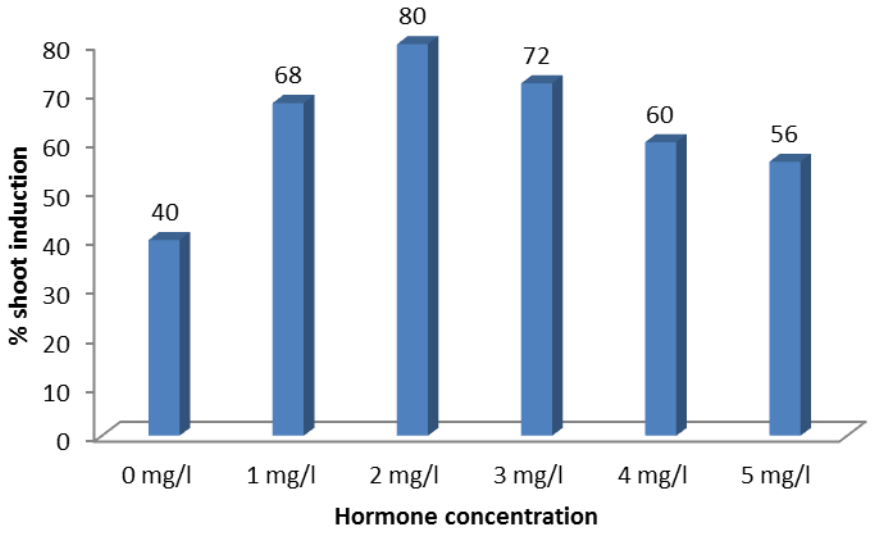

Figure 1.2 Effect of BA on percentage of shoot induction in Chrysanthemum

4.1.3 Number of shoot per explant

There was significant influence of different concentrations of BA on the number of shoots per explant. The treatment BA 2.0 mg/l gave the highest number of shoot (2.20, 2.60 and 3.00) at 14, 21 and 28 DAI (Days after induction), respectively (Table 1 and Plate 4) and the control treatment showed the lowest number of shoot (1.20, 1.40 and 1.60) at 14, 21 and 28 DAI (Table 1). Himstedt *et al.* (2001) studied regeneration from leaf and stem explants of 19 Chrysanthemum cultivars. Most of the cultivars regenerated shoots on MS medium supplemented with BAP 2.0 mg/1 with more than 10 regenerated shoots per explant in some of the cultivars. Waseem *et al.* (2009) carried out shoot multiplication of chrysanthemum using MS media supplemented with 1.0 mg/l BAP which showed maximum number of shoot (4.1) per explants. The findings of their study seems completely different from our result. This variation may be because of interaction between genotype, growth regulators and environmental factors. (Sen, J. *et al*., 2002).

Table 1. Effect of different concentration of BA on number of shoot at different DAI (Days after induction)

BA (mg/l)	Number of shoot/explants		
	14 DAI	21 DAI	28 DAI
0	1.20c	1.40c	1.60d
1.0	1.60b	2.20b	2.60b
2.0	2.20a	2.60a	3.00a
3.0	1.60b	2.00b	2.20c
4.0	1.40bc	1.60c	1.80d
5.0	1.20c	2.00b	2.20c
CV (%)	32.61	26.26	23.83
$LSD_{(0.05)}$	0.291	0.337	0.376

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

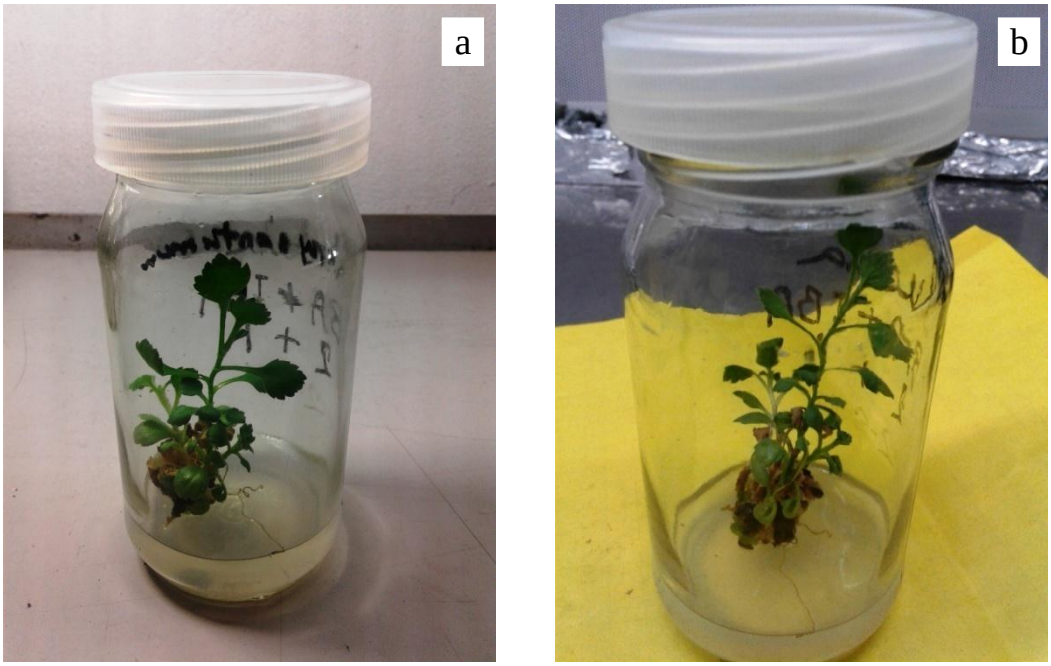

Plate 4. Highest number of shoot induction at (a) 21 DAI and (b) 28 DAI in 2.0 mg/l of BA treatment

4.1.4 Length of shoot

There was significant influence of different concentrations of BA at 5% level on the length of shoot. The highest length of shoot (1.34 cm, 2.60 cm and 3.12 cm) at 14, 21 and 28 DAI (Plate 5) was found in 3.0 mg/l BA and second highest length (1.14 cm, 2.18 cm and 2.90 cm) with 1.0 mg/l BA at 14, 21 and 28 DAI (Table 2). The control treatment gave the lowest length (2.08 cm) of shoot which was statistically similar with 5.0 mg/l (2.14 cm) at 28 DAI (Table 2).Waseem *et al.* (2009) carried out shoot regeneration of Chrysanthemum from shoot tip explants using MS media fortified with 1.0 mg/l BAP produced 5.0 cm length of shoots. This result partially supported present findings.

Table 2. Effect of different concentration of BA on length of shoot at different days after induction (DAI)

BA (mg/l)	Length of shoot (cm)		
	14 DAI	21 DAI	28 DAI
0	0.46f	0.96f	2.08e
1.0	1.14b	2.18b	2.90b
2.0	0.82c	1.66c	2.58c
3.0	1.34a	2.60a	3.12a
4.0	0.66d	1.54d	2.36d
5.0	0.60e	1.36e	2.14e
CV (%)	5.77	3.68	4.89
LSD (0.05)	0.053	0.082	0.159

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 5. Highest length of shoot induction at 28 DAI in the treatment 3.0 mg/l BA

4.1.5 Number of leaves

The different concentrations of BA showed significant variation on the number of leaves. The highest number of leaves (3.60, 6.20 and 9.60) at 14, 21 and 28 DAI (plate 6), respectively was noticed from the 2.0 mg/l BA which was statistically different from rest of others. Whereas the lowest number of leaves (1.40, 1.80 and 2.40) at 14, 21 and 28 DAI, respectively were noticed in control treatment (Table 3). Waseem *et al.* (2009) carried out an experiment of chrysanthemum from shoot tip explant using MS media supplemented with 1.0 mg/l BAP producing highest number (11.0) of leaves.

Table 3. Effect of different concentration of BA on number of leaves at different days after induction

BA (mg/ l)	Number of leaves		
	14 DAI	21 DAI	28 DAI
0	1.40c	1.80c	2.40f
1.0	3.20a	3.80b	7.40b
2.0	3.60a	6.20a	9.60a
3.0	2.40b	3.20b	6.20c
4.0	2.40b	3.60b	5.00d
5.0	2.20b	3.20b	3.80e
CV (%)	20.38	18.80	15.60
LSD (0.05)	0.533	0.674	1.011

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

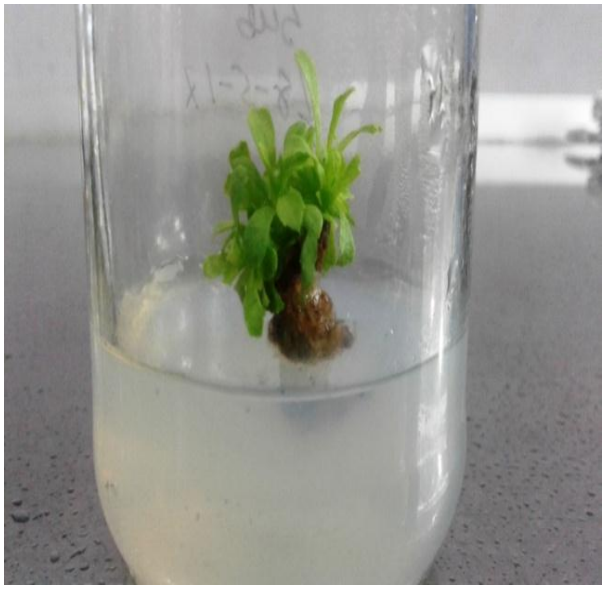

Plate 6. The treatment BA 2.0 mg/l showed the maximum number of leaves at 28 DAI.

4.2 Sub-experiment 2. Effect of BA and 2,4-D on callus and shoot induction potentiality in Chrysanthemum

From previous experiment, it was found that BA 2.0 mg/l gave the highest response in most of the parameters under studied. So, in this sub-experiment, BA 2.0 mg/l was used with 2,4-D (0.5, 1.0, 1.5 and 2.0 mg/l), respectively to know the callus and shoot induction performance. The result of the effect of different concentrations of BA with 2,4-D has been presented under following headings with Figure (2.1-2.3) and Table (4-5).

4.2.1 Days to callus initiation

Significant variations were observed among different concentrations of 2,4-D on days to callus initiation. The maximum days (19.80) to callus initiation were recorded in BA 2.0 mg/l + 2,4-D 2.0 mg/l treatment while minimum (14.20 days) was in BA 2.0 mg/l + 2,4-D 1.0 mg/l treatment (Figure 2.1). No callus was observed in control treatment. Rajlakshmi *et al.* (2013) studied that callus formation was obtained from leaf within 14.00 days in media supplemented with IAA and BAP. This result partially supported our findings.

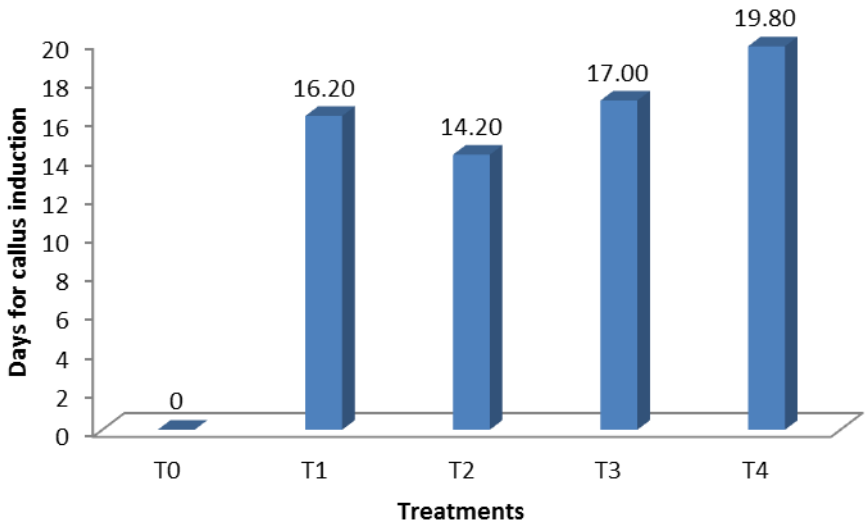

Figure 2.1 Effect of BA and 2,4-D on days for callus induction in Chrysanthemum

T_0 (Control)
T_1 (BA 2.0 mg/l+ 2,4-D 0.5 mg/l)
T_2 (BA 2.0 mg/l+ 2,4-D 1.0 mg/l)
T_3 (BA 2.0 mg/l+ 2,4-D 1.5 mg/l)
T_4 (BA 2.0 mg/l+ 2,4-D 2.0 mg/l)

4.2.2 Percentage of callus initiation

The treatment BA 2.0 mg/l+ 2,4-D 1.0 mg/l had produced the highest frequency of callus (76.00%), while the lowest percentage (68.00%) of callus was recorded at BA 2.0 mg/l + 2,4-D 2.0 mg/l (Fig. 2.2 and Plate 7). Callus was not observed in control treatment. Our findings are also in conformation to the results obtained by Hussain *et al.* (1994), who found chrysanthemum callus induction from seedling explants. Bhattacharya *et al.* (1990) studied the influence of different growth regulators on the *in vitro* morphogenesis of chrysanthemum. They reported that a combination of 0.1 mg/l IAA and 0.2 mg/l BAP was most appropriate for callus formation.

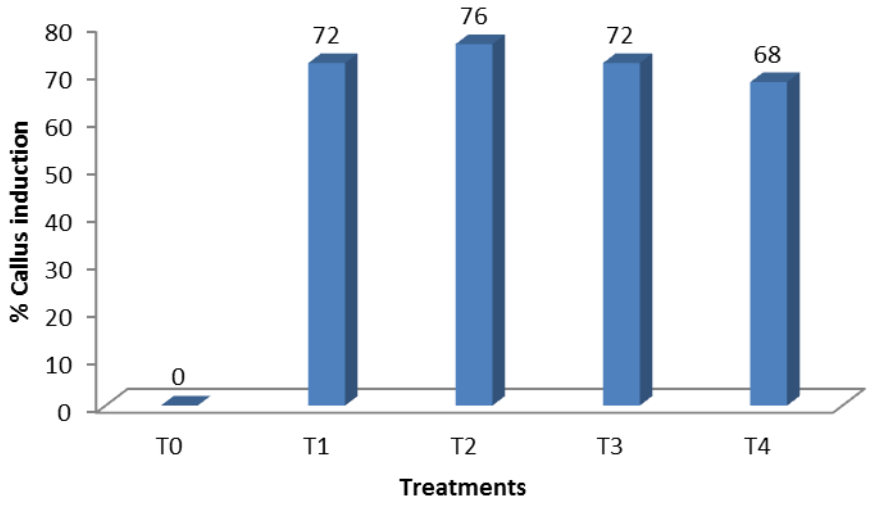

Figure 2.2 Effect of BA and 2,4-D on percentage of callus induction in Chrysanthemum

T_0 (Control)
T_1 (BA 2.0 mg/l+ 2,4-D 0.5 mg/l)
T_2 (BA 2.0 mg/l+ 2,4-D 1.0 mg/l)
T_3 (BA 2.0 mg/l+ 2,4-D 1.5 mg/l)
T_4 (BA 2.0 mg/l+ 2,4-D 2.0 mg/l)

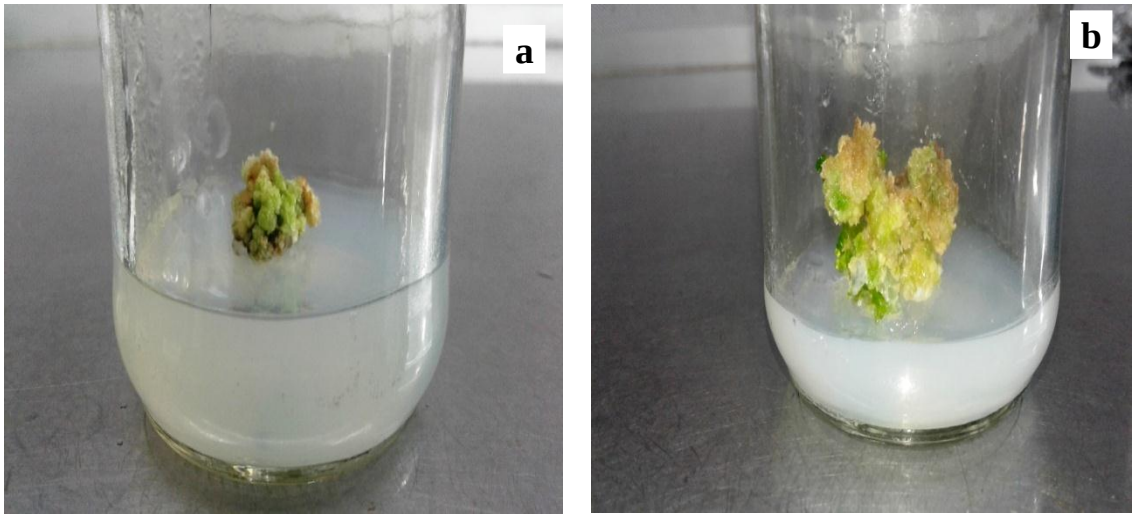

Plate 7. Effect of BA 2.0 mg/l and 2,4-D 1.0 mg/l for callus induction in chrysanthemum at (a) 30 DAI and (b) 40 DAI.

4.2.3 Weight of callus

Significant variations of different concentrations of BA and 2,4-D on the weight of callus were observed at 5% level of significance. The highest weight of callus (1.80 g, 2.70 g and 3.50 g) at 20, 30 and 40 DAI, respectively was noticed from the BA 2.0 mg/l + 2,4-D 1.0 mg/l which was statistically different from rest of others. Whereas the lowest weight of callus (0.79 g, 1.41g and 1.90 g) at 20, 30 and 40 DAI was found from BA 2.0 mg/l+ 2,4-D 2.0 mg/l (Table 4). It indicated that, higher dose of 2,4-D showed negative effect on callus formation.

Table 4. Effect of BA and 2,4-D on fresh weight of callus at different DAI

Treatment	Weight of callus		
	20 DAI	30 DAI	40 DAI
T_0(Control)	-	-	-
T_1 (BA 2.0 mg/l+ 2,4-D 0.5 mg/l)	1.50b	2.20b	2.70b
T_2 (BA 2.0 mg/l+ 2,4-D 1.0 mg/l)	1.80a	2.70a	3.50a
T_3 (BA 2.0 mg/l+ 2,4-D 1.5 mg/l)	1.20c	1.69c	2.29c
T_4 (BA 2.0 mg/l+ 2,4-D 2.0 mg/l)	0.79d	1.41d	1.90d
CV (%)	2.99	4.09	4.02
LSD (0.05)	0.042	0.083	0.041

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

4.2.4 Days to shoot initiation from callus

Significant variations were observed among different concentrations of BA and 2,4-D on days to shoot initiation from regenerated callus. The maximum days (17.40) to shoot initiation were recorded in BA 2.0 mg/l + 2,4-D 2.0 mg/l treatment where minimum days (9.20) was needed treatment BA 2.0 mg/l + 2,4-D 1.0 mg/l (Figure 2.3).

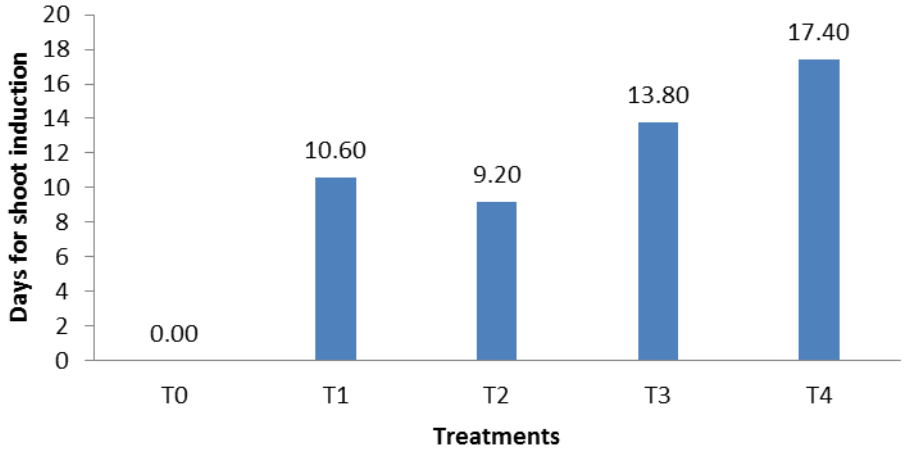

Figure 2.3 Effect of BA and 2,4-D on days for shoot induction from callus in Chrysanthemum

T_0 (Control)
T_1 (BA 2.0 mg/l+ 2,4-D 0.5 mg/l)
T_2 (BA 2.0 mg/l+ 2,4-D 1.0 mg/l)
T_3 (BA 2.0 mg/l+ 2,4-D 1.5 mg/l)
T_4 (BA 2.0 mg/l+ 2,4-D 2.0 mg/l)

4.2.5 Number of shoot

There was a significant difference observed on the number of shoot due to different concentrations of BA and 2,4-D . The highest number of shoot (1.80, 2.60 and 3.20) at 20, 30 and 40 DAI, respectively was obtained from the BA 2.0 mg/l + 2,4-D 1.0 mg/l which was statistically different from rest of others (Plate 8) whereas the lowest number of shoot (1.40, 1.60 and 2.20) was found from BA 2.0 mg/l + 2,4-D 2.0 mg/l at 20, 30 and 40 DAI. Mishra *et al.* (2006) obtained 4.43 shoots on shoot tip explants in MS medium supplemented with 3.0 mg/1 BA and 0.01 mg/l NAA and Sun *et al.* (2007) also reported best shoot regeneration from leaf explants via callus induction in medium with 0.2 mg/l NAA and 1 mg/1 BA chrysanthemum.

Table 5. Effect of BA and 2,4-D on number of shoot at different DAI

Treatment	Number of shoot		
	20 DAI	**30 DAI**	**40 DAI**
T_0 (control)	-	-	-
T_1 (BA 2.0 mg/l+ 2,4-D 0.5 mg/l)	1.60b	2.40a	2.80ab
T_2 (BA 2.0 mg/l+ 2,4-D 1.0 mg/l)	1.80a	2.60a	3.20a
T_3 (BA 2.0 mg/l+ 2,4-D 1.5 mg/l)	1.60b	2.20a	2.40bc
T_4 (BA 2.0 mg/l+ 2,4-D 2.0 mg/l)	1.40c	1.60b	2.20c
CV (%)	36.64	26.56	29.08
LSD (0.05)	0.194	0.457	0.559

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT.
CV= Coefficient of variation, LS= Level of significance, $LSD_{(0.05)}$= Least significant difference.

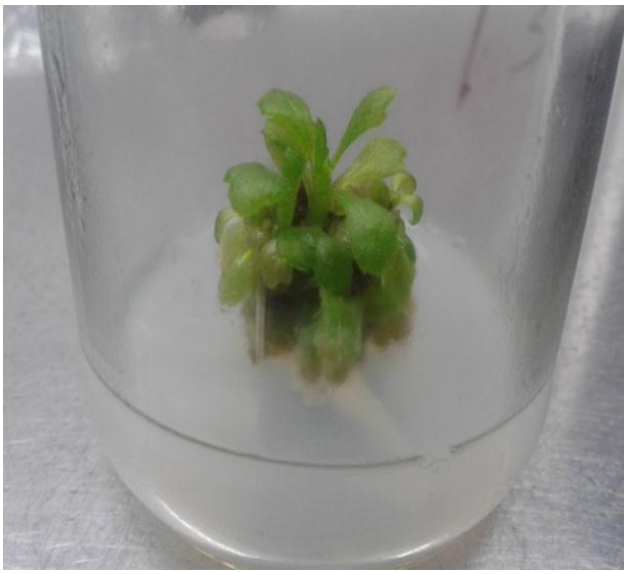

Plate 8. Effect of BA 2 mg/l and 2,4-D 1 mg/l on number of shoot initiation from callus of chrysanthemum at 40 DAI.

4.3 Sub-experiment 3. Effect of IAA on root induction potentiality in Chrysanthemum

The result of the effect of different concentrations of IAA has been presented under following headings with Figure (3.1-3.2) and Table (6-7).

4.3.1 Days to root initiation

Significant variations were observed among different concentrations of IAA on days to root induction. The maximum days (26.60) to root induction were recorded in control treatment followed by 2.5 mg/l (20.60 days) and 2.0 mg/l (17.80 days) of IAA. Besides, minimum 13.00 days were needed in 0.5 mg/l IAA followed by 1.0 mg/l (13.80 days) and 1.5 mg/l (15.80 days) (Figure 3.1). Nalini (2012) revealed that minimum 19.19 days were needed for root initiation through shoot tip explants in MS medium supplemented with IBA 1.0 mg/l. Present results partially supported by the author findings. This variation may be due to explants type, seasonal variations and environmental factors (Sen, J. *et al*., 2002).

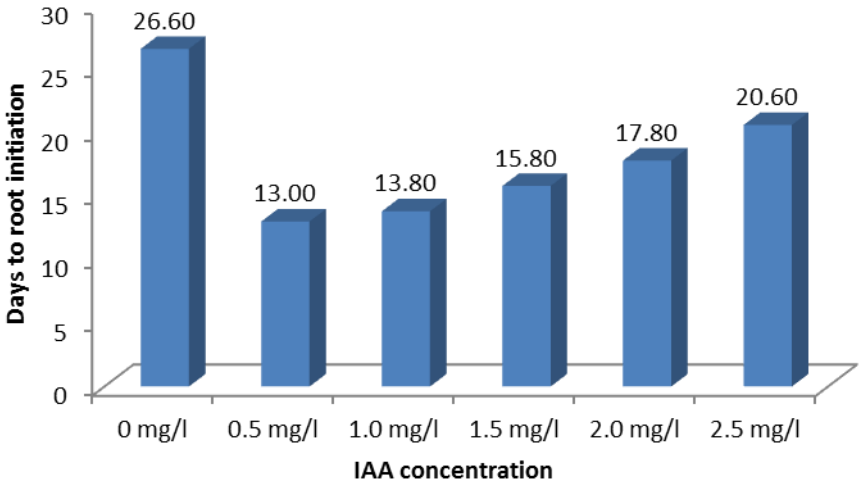

Figure 3.1 Effect of IAA on days to root induction in Chrysanthemum

4.3.2 Percentage of root initiation

The maximum percentage of root (80.00%) was obtained in 0.5 mg/l IAA treatment, while the minimum percentage (44.00%) of root initiation was in control treatment (Figure 3.2). Besides, it was noticed that with increasing IAA concentration, percent of root were decreased (Figure 3.2). Similar results on chrysanthemum were obtained by Long *et al*. (2006) and Karim *et al*. (2002). Shatanawi *et al.* (2010) also achieved *in vitro* rooting successfully on MS medium supplemented with IBA (0.2 mg/1) in chrysanthemum.

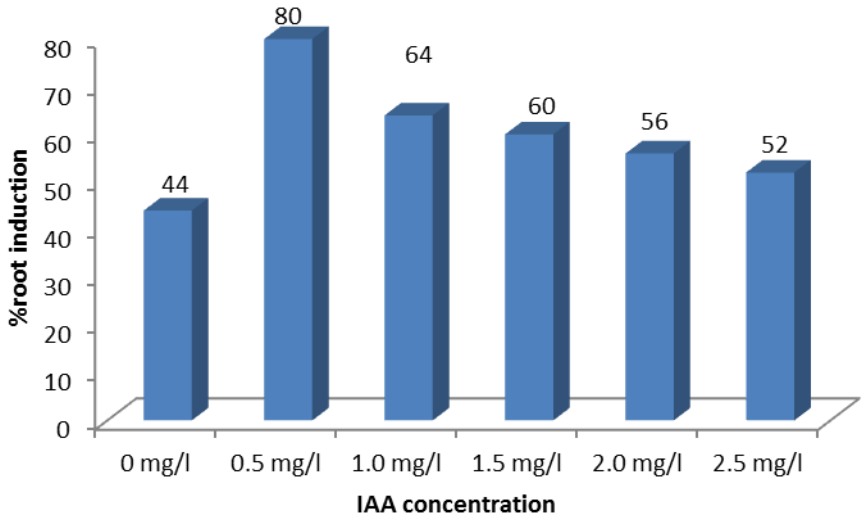

Figure 3.2 Effect of IAA on percentage of root induction in Chrysanthemum

4.3.3 Number of root per explant

There was significant influence of different concentrations of IAA on the number of roots per explant. The treatment IAA 1.5 mg/l gave the highest number of root (2.60, 3.60 and 5.40) at 14, 21 and 28 DAI, respectively (Plate 9 and Table 6) and the control treatment showed the lowest number of root (1.20, 1.60 and 1.80) at 14, 21 and 28 DAI, respectively (Table 6).

Table 6. Effect of different concentration of IAA on number of root at different DAI

IAA (mg/l)	Number of root/explants		
	14 DAI	21 DAI	28 DAI
0	1.20d	1.60c	1.80d
0.5	1.80bc	2.40b	3.60b
1.0	2.20ab	2.60b	4.20b
1.5	2.60a	3.60a	5.40a
2.0	1.40cd	1.80c	2.60c
2.5	1.40cd	1.80c	2.40cd
CV (%)	28.30	22.45	19.75
LSD (0.05)	0.505	0.533	0.630

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 9. Maximum number of root formation at 28 DAI in the treatment 1.5 mg/l of IAA in Chrysanthemum.

4.3.4 Length of root

There was meaningful influence of different concentrations of IAA on the length of root at 5% level of significance at laboratory condition. The highest length of root (2.16 cm, 2.76 cm and 3.16 cm) at 14, 21 and 28 DAI, respectively was found in 1.0 mg/l IAA (Plate 10 and Table 7). The control treatment found the lowest number of root (0.42 cm, 0.80 cm and 1.18 cm) at 14, 21 and 28 DAI, respectively (Table 7). Labade *et al.* (2016) found superiority of ½ MS + 0.2 mg/l IBA as compared to all the other treatments as it produced largest roots (9±0.19 cm) of Chrysanthemum.

Table 7. Effect of different concentration of IAA on length of root at different days after induction

IAA (mg/l)	Length of root (cm)		
	14 DAI	21 DAI	28 DAI
0	0.42f	0.80f	1.18f
0.5	1.68b	2.64b	3.00b
1.0	2.16a	2.76a	3.16a
1.5	1.14c	2.12c	2.74c
2.0	0.78d	1.76d	2.30d
2.5	0.66e	1.40e	1.76e
CV (%)	7.06	5.48	4.62
LSD (0.05)	0.136	0.139	0.143

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT.
CV= Coefficient of variation, LS= Level of significance, $LSD_{(0.05)}$= Least significant difference.

Plate 10. Effect of IAA 1.0 mg/l on highest length of root measured at 28 DAI

4.4 Sub-experiment 4. Combined effect of BA and IAA on shoot and root induction potentiality in Chrysanthemum

The results of the combined effect of different concentrations of BA + IAA have been presented under following headings with Table 8-14.

4.4.1 Days to shoot initiation

Variations were observed among different concentrations of BA+IAA on days to shoot initiation. The maximum days (15.80 days) to shoot initiation was recorded in control and BA 2.0 mg/l+ IAA 1.0 mg/l required minimum (7.60 days) which was statistically similar with BA 2.0 mg/l+ IAA 0.5 mg/l (8.20 days) (Table 8).

4.4.2 Percentage of shoot initiation

Significant variation was observed for the combine used of BA+IAA on the percent of shoot initiation per explants. Maximum percentage (76.00%) of shoot induction was noticed in the treatment BA 2.0 mg/l + IAA 1.0 mg/l which is statistically similar (76.00%) with BA 2.0 mg/l + IAA 0.5 mg/l treatment whereas minimum percentage (48.00%) initiation was recorded in control (hormone free media) (Table 8). This result was partially supported by Bo *et al.* (2005). They obtained adventitious shoots of Chrysanthemum of 93.8 % on MS media supplemented with 2.0 mg/1 BA and 2.0 mg/1 NAA. This variation may be occurred due to plant internal physiology and hormonal effect.

Table 8. Combined effect of different concentration of BA and IAA on days to shoot initiation and percent of shoot initiation

Treatment	Days to shoot initiation	Percent of shoot initiation
Control	15.80a	48.00f
BA 1.0 mg/l+ IAA0.5 mg/l	10.80ij	68.00a-c
BA 1.0 mg/l+ IAA1.0 mg/l	10.80ij	60.00c-e
BA 1.0 mg/l+ IAA1.5 mg/l	11.00ij	52.00ef
BA 1.0 mg/l+ IAA2.0 mg/l	11.60hi	64.00b-d
BA 1.0 mg/l+ IAA2.5 mg/l	11.20i	68.00b-d
BA 2.0 mg/l+ IAA0.5 mg/l	8.20l	76.00a
BA 2.0 mg/l+ IAA1.0 mg/l	7.60l	76.00a
BA 2.0 mg/l+ IAA 1.5 mg/l	9.40k	72.00ab
BA 2.0 mg/l+ IAA 2.0 mg/l	9.80jk	68.00a-c
BA 2.0 mg/l+ IAA2.5 mg/l	11.20i	64.00b-d
BA 3.0 mg/l+ IAA0.5 mg/l	12.60f-h	60.00c-e
BA 3.0 mg/l+ IAA1.0 mg/l	12.60f-h	68.00a-c
BA 3.0 mg/l+ IAA1.5 mg/l	12.80f-h	72.00ab
BA 3.0 mg/l+ IAA2.0 mg/l	13.20d-g	64.00b-d
BA 3.0 mg/l+ IAA2.5 mg/l	13.40c-f	60.00c-e
BA 4.0 mg/l+ IAA0.5 mg/l	13.80b-f	60.00c-e
BA 4.0 mg/l+ IAA1.0 mg/l	12.00g-i	60.00c-e
BA 4.0 mg/l+ IAA 1.5 mg/l	13.00e-g	52.00ef
BA 4.0 mg/l+ IAA2.0 mg/l	13.60b-f	56.00d-f
BA 4.0 mg/l+ IAA2.5 mg/l	14.80ab	56.00d-f
BA 5.0 mg/l+ IAA0.5 mg/l	14.60a-c	48.00f
BA 5.0 mg/l+ IAA1.0 mg/l	13.80b-f	48.00f
BA 5.0 mg/l+ IAA1.5 mg/l	14.20b-e	48.00f
BA 5.0 mg/l+ IAA2.0 mg/l	14.60a-c	48.00f
BA 5.0 mg/l+ IAA2.5 mg/l	14.40b-d	48.00f
CV (%)	7.14	19.67
$LSD_{(0.05)}$	1.156	7.932

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

4.4.3 Number of shoot per explant

There was significant influence of combined concentrations of BA and IAA on the number of shoot per explant at 14, 21 and 28 days after induction. The treatment BA 2.0 mg/l+ IAA 1.0 mg/l gave the highest number of shoot (2.20, 2.80 and 3.40) at 14, 21 and 28 DAI respectively (Plate 11) whereas the lowest number of shoot (1.00, 1.60 and 1.80) at 14, 21 and 28 DAI, respectively was found with hormone free media (Table 9).

Similarly, when the combination of different concentrations of IAA and BAP were used, significant results regarding to the number of shoots regeneration of chrysanthemum plantlets from nodal segments were achieved by Waseem *et al.* (2009).

Table 9. Combined effect of BA and IAA on number of shoot

Treatment	Number of Shoot/explants		
	14 DAI	**21 DAI**	**28 DAI**
Control	1.00d	1.60de	1.80gh
BA 1.0 mg/l+ IAA0.5 mg/l	1.60bc	2.40ab	2.60c-e
BA 1.0 mg/l+ IAA1.0 mg/l	1.80b	2.40ab	3.00a-c
BA 1.0 mg/l+ IAA1.5 mg/l	1.40b-d	2.00b-d	2.80b-d
BA 1.0 mg/l+ IAA2.0 mg/l	1.60bc	2.20bc	3.20ab
BA 1.0 mg/l+ IAA2.5 mg/l	1.40b-d	2.20bc	3.20ab
BA 2.0 mg/l+ IAA0.5 mg/l	1.80b	2.40ab	3.00a-c
BA 2.0 mg/l+ IAA1.0 mg/l	2.20a	2.80a	3.40a
BA 2.0 mg/l+ IAA 1.5 mg/l	1.60bc	2.40ab	3.00a-c
BA 2.0 mg/l+ IAA 2.0 mg/l	1.40b-d	2.20bc	2.60c-e
BA 2.0 mg/l+ IAA2.5 mg/l	1.40b-d	1.60de	2.20e-g
BA 3.0 mg/l+ IAA0.5 mg/l	1.40b-d	1.60de	2.20e-g
BA 3.0 mg/l+ IAA1.0 mg/l	1.20cd	1.60de	2.20e-g
BA 3.0 mg/l+ IAA1.5 mg/l	1.40b-d	1.60de	2.40d-f
BA 3.0 mg/l+ IAA2.0 mg/l	1.60bc	1.80c-d	2.20e-g
BA 3.0 mg/l+ IAA2.5 mg/l	1.40b-d	1.40e	2.00f-h
BA 4.0 mg/l+ IAA0.5 mg/l	1.20cd	1.60de	1.80gh
BA 4.0 mg/l+ IAA1.0 mg/l	1.40b-d	1.60de	2.20e-g
BA 4.0 mg/l+ IAA 1.5 mg/l	1.20cd	1.40e	2.20e-g
BA 4.0 mg/l+ IAA2.0 mg/l	1.40b-d	1.80c-e	2.20e-g
BA 4.0 mg/l+ IAA2.5 mg/l	1.40b-d	2.00b-d	2.00f-h
BA 5.0 mg/l+ IAA0.5 mg/l	1.20cd	1.40e	1.80gh
BA 5.0 mg/l+ IAA1.0 mg/l	1.40b-d	1.40e	2.00f-h
BA 5.0 mg/l+ IAA1.5 mg/l	1.40b-d	1.60de	1.60h
BA 5.0 mg/l+ IAA2.0 mg/l	1.20cd	1.60de	1.60h
BA 5.0 mg/l+ IAA2.5 mg/l	1.40b-d	1.40e	1.80gh
CV (%)	36.58	31.25	25.22
$LSD_{(0.05)}$	0.512	0.453	0.487

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

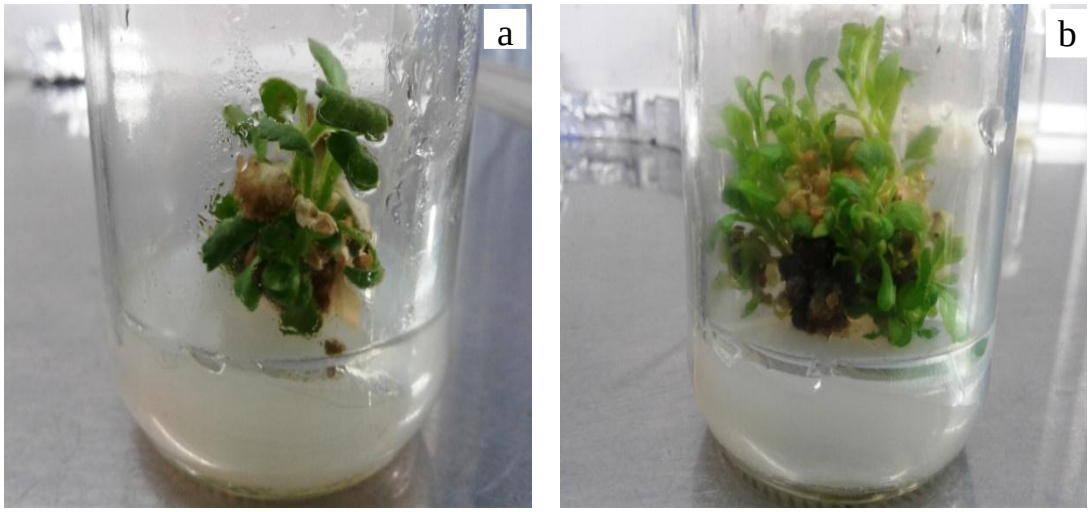

Plate 11. Maximum Number of shoot observed at (a) 21 DAI and (b) 28 DAI in the treatment BA 2.0 mg/l+ IAA 1.0 mg/l

4.4.4 Length of shoot (cm)

With different concentrations of BA and IAA, significant influence was found on the average length of shoot. The maximum average length of shoot (2.48 cm, 2.84 cm and 3.66 cm) at 14, 21 and 28 DAI was noticed from the BA 2.0 mg/l + IAA 1.0 mg/l which were statistically different from rest of others (Plate 12). It was the minimum (0.40 cm, 0.66 cm and 1.08 cm) at 14, 21 and 28 DAI in control (Table 10). Labade *et al.* (2016) found that MS medium containing 1.0 mg/l BAP + 0.1 mg/l IAA, shown 90% shoot initiation and 5.5 ± 0.51 cm average length of shoot per explants in Chrysanthemum. The results of our experiment were varied may be due to genotype and culture environment. (Sen, J. *et al*., 2002).

Table 10. Combined effect of different concentration of BA and IAA on length of shoot

Treatments	Length of shoot (cm)		
	14 DAI	**21 DAI**	**28 DAI**
Control	0.40k	0.66m	1.08m
BA 1.0 mg/l+ IAA0.5 mg/l	2.10b	2.32cd	2.56c
BA 1.0 mg/l+ IAA1.0 mg/l	1.84cd	2.24c-e	2.48c
BA 1.0 mg/l+ IAA1.5 mg/l	1.70de	2.16d-f	2.32de
BA 1.0 mg/l+ IAA2.0 mg/l	1.58ef	2.08ef	2.20ef
BA 1.0 mg/l+ IAA2.5 mg/l	1.50f	2.02f	2.12f
BA 2.0 mg/l+ IAA0.5 mg/l	2.36a	2.62b	3.14b
BA 2.0 mg/l+ IAA1.0 mg/l	2.48a	2.84a	3.66a
BA 2.0 mg/l+ IAA 1.5 mg/l	2.10b	2.36c	3.02b
BA 2.0 mg/l+ IAA 2.0 mg/l	2.02bc	2.28cd	2.54c
BA 2.0 mg/l+ IAA2.5 mg/l	1.92bc	2.20c-e	2.44cd
BA 3.0 mg/l+ IAA0.5 mg/l	1.52ef	1.86g	2.06f
BA 3.0 mg/l+ IAA1.0 mg/l	1.4fe	1.74g	1.90g
BA 3.0 mg/l+ IAA1.5 mg/l	1.40f	1.56h	1.74h
BA 3.0 mg/l+ IAA2.0 mg/l	0.98g	1.38i	1.56i
BA 3.0 mg/l+ IAA2.5 mg/l	0.82g-j	1.22ij	1.46i-k
BA 4.0 mg/l+ IAA0.5 mg/l	0.68ij	1.00kl	1.32kl
BA 4.0 mg/l+ IAA1.0 mg/l	0.72h-j	1.06j-l	1.32kl
BA 4.0 mg/l+ IAA 1.5 mg/l	0.60j	1.04kj	1.22lm
BA 4.0 mg/l+ IAA2.0 mg/l	0.84g-i	1.16jk	1.40jk
BA 4.0 mg/l+ IAA2.5 mg/l	0.92gh	1.34i	1.50ij
BA 5.0 mg/l+ IAA0.5 mg/l	0.74h-j	1.14jk	1.34kl
BA 5.0 mg/l+ IAA1.0 mg/l	0.76h-j	0.94l	1.16m
BA 5.0 mg/l+ IAA1.5 mg/l	0.68ij	1.00kl	1.16m
BA 5.0 mg/l+ IAA2.0 mg/l	0.66ij	0.96l	1.22lm
BA 5.0 mg/l+ IAA2.5 mg/l	0.60j	0.92l	1.10m
CV (%)	11.16	9.99	7.85
$LSD_{(0.05)}$	0.177	0.158	0.137

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 12. Effect of BA 2.0 mg/l+ IAA 1.0 mg/l on length of shoot observed at 28 DAI.

4.4.5 Number of leaves

There was significant influence of different concentrations of BA on the number of leaves. The highest number of leaves (6.20, 8.20 and 11.60) at 14, 21 and 28 days after induction, respectively was noticed from the BA 3.0 mg/l + IAA 1.0 mg/l which showed better performance from rest of others (Plate 13). Whereas the lowest average number of leaves (1.20, 1.60 and 2.20) at 14, 21 and 28 DAI, respectively were noticed in control treatment (Table 11).

Table 11. Combined effect of different concentration of BA and IAA on number of leaves

Treatments	Number of leaves		
	14 DAI	21 DAI	28 DAI
Control	1.20j	1.60k	2.20l-n
BA 1.0 mg/l+ IAA 0.5 mg/l	4.00bc	6.00b	7.60cd
BA 1.0 mg/l+ IAA 1.0 mg/l	4.00bc	5.80bc	7.00de
BA 1.0 mg/l+ IAA 1.5 mg/l	3.60cd	5.20cd	6.40ef
BA 1.0 mg/l+ IAA 2.0 mg/l	3.60cd	4.40b	6.20ef
BA 1.0 mg/l+ IAA 2.5 mg/l	3.20de	5.20a	6.40d-f
BA 2.0 mg/l+ IAA 0.5 mg/l	4.40b	6.00bc	9.80b
BA 2.0 mg/l+ IAA 1.0 mg/l	2.40fg	3.60gh	4.60g-i
BA 2.0 mg/l+ IAA 1.5 mg/l	4.20b	5.40bc	8.40c
BA 2.0 mg/l+ IAA 2.0 mg/l	3.40d	4.60de	6.40d-f
BA 2.0 mg/l+ IAA 2.5 mg/l	2.80ef	3.80fg	5.60fg
BA 3.0 mg/l+ IAA 0.5 mg/l	2.60fg	4.00f-g	4.80gh
BA 3.0 mg/l+ IAA 1.0 mg/l	6.20a	8.20a	11.60a
BA 3.0 mg/l+ IAA 1.5 mg/l	2.60fg	3.40g-i	4.00h-j
BA 3.0 mg/l+ IAA 2.0 mg/l	2.80ef	3.00h-j	4.00h-j
BA 3.0 mg/l+ IAA 2.5 mg/l	2.40fg	2.80ij	3.60h-k
BA 4.0 mg/l+ IAA 0.5 mg/l	2.20gh	2.80ij	3.20j-l
BA 4.0 mg/l+ IAA 1.0 mg/l	2.20gh	2.80ij	3.40i-l
BA 4.0 mg/l+ IAA 1.5 mg/l	2.20gh	2.60j	3.40i=l
BA 4.0 mg/l+ IAA 2.0 mg/l	1.80hi	3.00h-j	4.00h-j
BA 4.0 mg/l+ IAA 2.5 mg/l	1.80hi	2.80ij	3.40i-l
BA 5.0 mg/l+ IAA 0.5 mg/l	1.80hi	2.60j	3.00j-m
BA 5.0 mg/l+ IAA 1.0 mg/l	1.60ij	2.40j	2.40k-n
BA 5.0 mg/l+ IAA 1.5 mg/l	1.60ij	2.40j	2.40k-n
BA 5.0 mg/l+ IAA 2.0 mg/l	1.40ij	1.60k	1.80mn
BA 5.0 mg/l+ IAA 2.5 mg/l	1.20j	1.20k	1.40n
CV (%)	21.96	20.52	18.27
LSD (0.05)	0.455	0.673	1.119

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 13. Effect of BA 3.0 mg/l + IAA 1.0 mg/l showing number of leaves observed at 28 DAI.

4.4.6 Days to root induction

Significant variation was observed among different concentrations of BA and IAA on days to root induction. The maximum days (29.80 days) to root induction was recorded in control treatment and minimum days (12.20 days) was required in BA 2.0 mg/l + IAA 1.0 mg/l concentration (Table 12). Nalini (2012) cultured shoot tip explants in MS medium containing kinetin 3.0 mg/1 + IAA 2.0 mg/1 and found good percentage of root in 17.91 days (minimum).

4.4.7 Percentage of root induction

Variations were observed among different concentrations of BA and IAA on percent of explants showing root induction. The highest percentage (76.00%) of root induction was recorded with BA 3.0 mg/l+ IAA 1.5 mg/l. The treatment BA 2.0 mg/l + IAA 0.5 mg/l, BA 2.0 mg/l+ IAA 1.0 mg/l and BA 2.0 mg/l+ IAA 2.5 mg/l showed statistically similar percent (68.00%) followed by best result whereas the lowest percentage (24.00%) of root induction was recorded in control condition (Table 12). Nalini (2012) cultured shoot tip explants in MS medium containing kinetin 3.0 mg/1 + IAA 2.0 mg/1 and reported it to be the best treatment combination as it produced 67.82 per cent.

Table 12. Combined effect of BA and IAA on Days to root induction and percent of root initiation of roots

Treatments	Days to root induction	Percentage of root initiation
Control	29.80a	24.00g
BA1.0 mg/l+ IAA 0.5 mg/l	17.60jk	64.00bc
BA 1.0 mg/l+ IAA 1.0 mg/l	18.00ij	64.00bc
BA 1.0 mg/l+ IAA 1.5 mg/l	17.60jk	56.00c-e
BA 1.0 mg/l+ IAA 2.0 mg/l	17.40jk	48.00ef
BA 1.0 mg/l+ IAA 2.5 mg/l	18.20h-j	56.00c-e
BA 2.0 mg/l+ IAA 0.5 mg/l	13.60m	68.00ab
BA 2.0 mg/l+ IAA 1.0 mg/l	12.20n	68.00ab
BA 2.0 mg/l+ IAA 1.5 mg/l	15.20l	60.00b-d
BA 2.0 mg/l+ IAA 2.0 mg/l	16.40kl	60.00b-d
BA 2.0 mg/l+ IAA 2.5 mg/l	17.00jk	68.00ab
BA 3.0 mg/l+ IAA 0.5 mg/l	19.20g-i	64.00bc
BA 3.0 mg/l+ IAA 1.0 mg/l	19.60f-h	60.00b-d
BA 3.0 mg/l+ IAA 1.5 mg/l	19.60gh	76.00a
BA 3.0 mg/l+ IAA 2.0 mg/l	19.40gh	60.00b-d
BA 3.0 mg/l+ IAA 2.5 mg/l	19.60gh	64.00bc
BA 4.0 mg/l+ IAA 0.5 mg/l	21.00ef	64.00bc
BA 4.0 mg/l+ IAA 1.0 mg/l	20.00e-g	60.00b-d
BA 4.0 mg/l+ IAA 1.5 mg/l	21.20e	60.00b-d
BA 4.0 mg/l+ IAA 2.0 mg/l	22.80d	52.00d-f
BA 4.0 mg/l+ IAA 2.5 mg/l	24.00cd	48.00ef
BA 5.0 mg/l+ IAA 0.5 mg/l	23.60cd	48.00ef
BA 5.0 mg/l+ IAA 1.0 mg/l	24.20c	48.00ef
BA 5.0 mg/l+ IAA 1.5 mg/l	24.80bc	44.00f
BA 5.0 mg/l+ IAA 2.0 mg/l	26.00b	44.00f
BA 5.0 mg/l+ IAA 2.5 mg/l	26.00b	44.00f
CV (%)	12.4	21.13
LSD (0.05)	1.273	8.231

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

4.4.8 Number of root per explant

There was significant influence of different concentrations of BA and IAA on the number of root per explant. The treatment BA 2.0 mg/l + IAA 1.0 mg/l gave the highest number of root (2.40, 3.20 and 4.20) at 14, 21 and 28 DAI (Plate 14), respectively whereas the lowest number of root (1.20, 1.60 and 2.00) at 14, 21 and 28 DAI, respectively was found with hormone free media (Table 13).

Table 13. Combined effects of BA and IAA on number of roots at different DAI

Treatments	Number of root/explants		
	14 DAI	21 DAI	28 DAI
Control	1.20d	1.60de	2.00h-j
BA 1.0 mg/l+ IAA 5 mg/l	2.00b	2.60b	4.00ab
BA 1.0 mg/l+ IAA 1.0 mg/l	2.20ab	2.60b	3.40cd
BA 1.0 mg/l+ IAA 1.5 mg/l	2.40a	2.20bc	3.40cd
BA 1.0 mg/l+ IAA 2.0 mg/l	2.20ab	2.40bc	2.80ef
BA 1.0 mg/l+ IAA 2.5 mg/l	1.60c	2.20bc	2.80ef
BA 2.0 mg/l+ IAA 0.5 mg/l	2.00b	2.60b	3.60bc
BA 2.0 mg/l+ IAA 1.0 mg/l	2.40a	3.20a	4.20a
BA 2.0 mg/l+ IAA 1.5 mg/l	1.60c	2.40bc	3.00de
BA 2.0 mg/l+ IAA 2.0 mg/l	1.40cd	1.60e	2.60e-g
BA 2.0 mg/l+ IAA 2.5 mg/l	1.60c	2.20bc	2.20g-i
BA 3.0 mg/l+ IAA 0.5 mg/l	1.40cd	2.20bc	2.40f-h
BA 3.0 mg/l+ IAA 1.0 mg/l	1.40cd	2.00cd	2.40f-h
BA 3.0 mg/l+ IAA 1.5 mg/l	1.40cd	1.60de	2.40f-h
BA 3.0 mg/l+ IAA 2.0 mg/l	1.40cd	1.60de	2.20g-i
BA 3.0 mg/l+ IAA 2.5 mg/l	1.40cd	1.60de	2.20g-i
BA 4.0 mg/l+ IAA 0.5 mg/l	1.20d	1.20e	1.60jk
BA 4.0 mg/l+ IAA 1.0 mg/l	1.40cd	1.40e	2.00h-j
BA 4.0 mg/l+ IAA 1.5 mg/l	1.20cd	1.60de	2.00h-j
BA 4.0 mg/l+ IAA 2.0 mg/l	1.20cd	1.40e	1.60jk
BA 4.0 mg/l+ IAA 2.5 mg/l	1.40cd	1.60de	1.80i-k
BA 5.0 mg/l+ IAA 0.5 mg/l	1.40cd	1.40e	1.40k
BA 5.0 mg/l+ IAA 1.0 mg/l	1.20cd	1.20e	1.40k
BA 5.0 mg/l+ IAA 1.5 mg/l	1.00d	1.20e	1.40k
BA 5.0 mg/l+ IAA 2.0 mg/l	1.20cd	1.40e	1.40k
BA 5.0 mg/l+ IAA 2.5 mg/l	1.20cd	1.40e	1.40k
CV (%)	33.75	29.23	26.31
LSD (0.05)	0.329	0.388	0.465

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 14. Effect of BA 2.0 mg/l+ IAA1.0 mg/l showing maximum number of root observed at 28 DAI.

4.4.9 Length of root (cm)

There was significant influence of different combined concentrations of BA and IAA on the length of root. The highest length of root (2.50 cm, 3.60 cm and 4.40 cm) at 14, 21 and 28 DAI (Plate 15), respectively was found in BA 2.0 mg/l+ IAA 1.0 mg/l (Table 14). The control treatment found the lowest number of root (0.38 cm, 0.62 cm and 1.04 cm) at 14, 21 and 28 DAI, respectively (Table 14).

Table 14. Combined effect of BA and IAA on length of root at different DAI

Treatments	Length of root		
	14 DAI	21 DAI	28 DAI
Control	0.38n	0.62p	1.04j
BA 1.0 mg/l+ IAA0.5 mg/l	2.14cd	3.16bgh	3.34bc
BA 1.0 mg/l+ IAA1.0 mg/l	1.74e	3.14bch	3.26bc
BA 1.0 mg/l+ IAA1.5 mg/l	1.72e	3.06b-d	3.18b-d
BA 1.0 mg/l+ IAA2.0 mg/l	1.66ef	2.94c-e	3.08b-e
BA 1.0 mg/l+ IAA2.5 mg/l	1.52f	2.80ef	3.00c-e
BA 2.0 mg/l+ IAA0.5 mg/l	2.24bc	3.44a	4.08a
BA 2.0 mg/l+ IAA1.0 mg/l	2.50a	3.60a	4.40a
BA 2.0 mg/l+ IAA 1.5 mg/l	2.34ab	3.48a	4.10a
BA 2.0 mg/l+ IAA 2.0 mg/l	1.98d	2.90de	3.74ab
BA 2.0 mg/l+ IAA2.5 mg/l	1.64ef	2.64f	3.18b-d
BA 3.0 mg/l+ IAA0.5 mg/l	1.30g	2.42g	2.72c-f
BA 3.0 mg/l+ IAA1.0 mg/l	1.22g	2.28gh	2.50d-g
BA 3.0 mg/l+ IAA1.5 mg/l	1.00h	2.10hi	2.28f-h
BA 3.0 mg/l+ IAA2.0 mg/l	0.88h-j	2.02ij	2.44e-g
BA 3.0 mg/l+ IAA2.5 mg/l	1.00h	1.88jk	2.22f-h
BA 4.0 mg/l+ IAA0.5 mg/l	0.84h-j	1.48lm	1.86g-i
BA 4.0 mg/l+ IAA1.0 mg/l	0.90hi	1.52lm	2.06f-h
BA 4.0 mg/l+ IAA 1.5 mg/l	0.72i-l	1.48lm	2.40f-h
BA 4.0 mg/l+ IAA2.0 mg/l	0.72i-l	1.70kl	2.14f-h
BA 4.0 mg/l+ IAA2.5 mg/l	0.68j-l	1.56l	2.00f-h
BA 5.0 mg/l+ IAA0.5 mg/l	0.76i-k	1.50lm	1.84g-i
BA 5.0 mg/l+ IAA1.0 mg/l	0.60k-m	1.30mn	1.58h-j
BA 5.0 mg/l+ IAA1.5 mg/l	0.54l-n	1.18no	1.64h-j
BA 5.0 mg/l+ IAA2.0 mg/l	0.42mn	1.00o	1.26ij
BA 5.0 mg/l+ IAA2.5 mg/l	0.46mn	1.02o	1.14ij
CV (%)	11.47	7.74	6.30
$LSD_{(0.05)}$	0.177	0.202	0.643

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 15. Effect of BA 2.0 mg/l+ IAA1.0 mg/l showing highest length of root observed at 28 DAI.

4.5 The comparative performance of growth hormone on shoot and root development are shown as following discussion (Table 15 and Table 16):

Table 15. Comparative performance of growth hormone in case of shoot morphology

Growth Hormone	Percent of shoot (%)	No. of shoot	Length of shoot (cm)
BA	80 (2.0 mg/l)	3.00 (2.0 mg/l)	3.12 (3.0 mg/l)
BA and IAA	76 (2.0 mg/l+ 1.0 mg/l)	3.40 (2.0 mg/l+ 1.0mg/l)	3.66 (2.0 mg/l+ 1.0 mg/l)

Table 16. Comparative performance of growth hormone in case of root morphology

Growth Hormone	Percent of root (%)	No. of root	Length of root (cm)
IAA	80 (0.5 mg/l)	5.40(1.5 mg/l)	3.16(1.0 mg/l)
BA and IAA	76 (3.0 mg/l+ 1.5 mg/l)	4.20 (2.0 mg/l+ 1.0 mg/l)	4.40 (2.0 mg/l+ 1.0 mg/l)

The results showed that combination of BA and IAA was better than BA alone in the basal MS medium in terms of number and length of shoot. But BA showed the better performance for percentage of shoot than the combine dose of BA and IAA (Table 15). The treatment IAA 0.5 mg/l and 1.5 mg/l alone gave the best result than combine dose of BA and IAA with (3.0 mg/l + 1.5 mg/l) and (2.0 mg/l+ 1.5 mg/l) in case of percentage of root and no. of root. On the other hand, combination of BA and IAA with 2.0 mg/l + 1.0 mg/l showed the good performance than BA 1.0 mg/l alone for length of root (Table 16).

Sub-experiment 5. *Ex vitro* acclimatization and establishment of plantlets on soil

After a satisfactory number of shoot and root development at 6-8 weeks of culture the individual plantlets were moved from vial carefully without any root damage. The roots were washed with running tap water for removing surplus media. The plantlets were then transplanted into small plastic pot prepared with a standard ratio of cowdung and soil in a shade condition. The plantlets were sprayed occasionally with water for maintaining humidity. At first 25 plants were transplanted, 20 survived in shade condition and survival rate was 80%. Finally in open atmospheric condition 20 plants were transplanted 15 survived (Table 17 and Plate 16) and survival rate was 75%. Sarkar and Shaheen (2009) carried out *in vitro* regeneration of chrysanthemum and plantlets were successfully established in soil after rooting (100%). So, analyzing the survival rate it can be said that acclimatization potentiality of Chrysanthemum was satisfactory.

Table 17. Survival rate of *in vitro* regenerated plantlets of Chrysanthemum

Acclimatization	No. of plants transplanted	No. of plants survived	Percentage of survival rate
In shade house with controlled atmosphere	25	20	80
In open atmospheric area	20	15	75

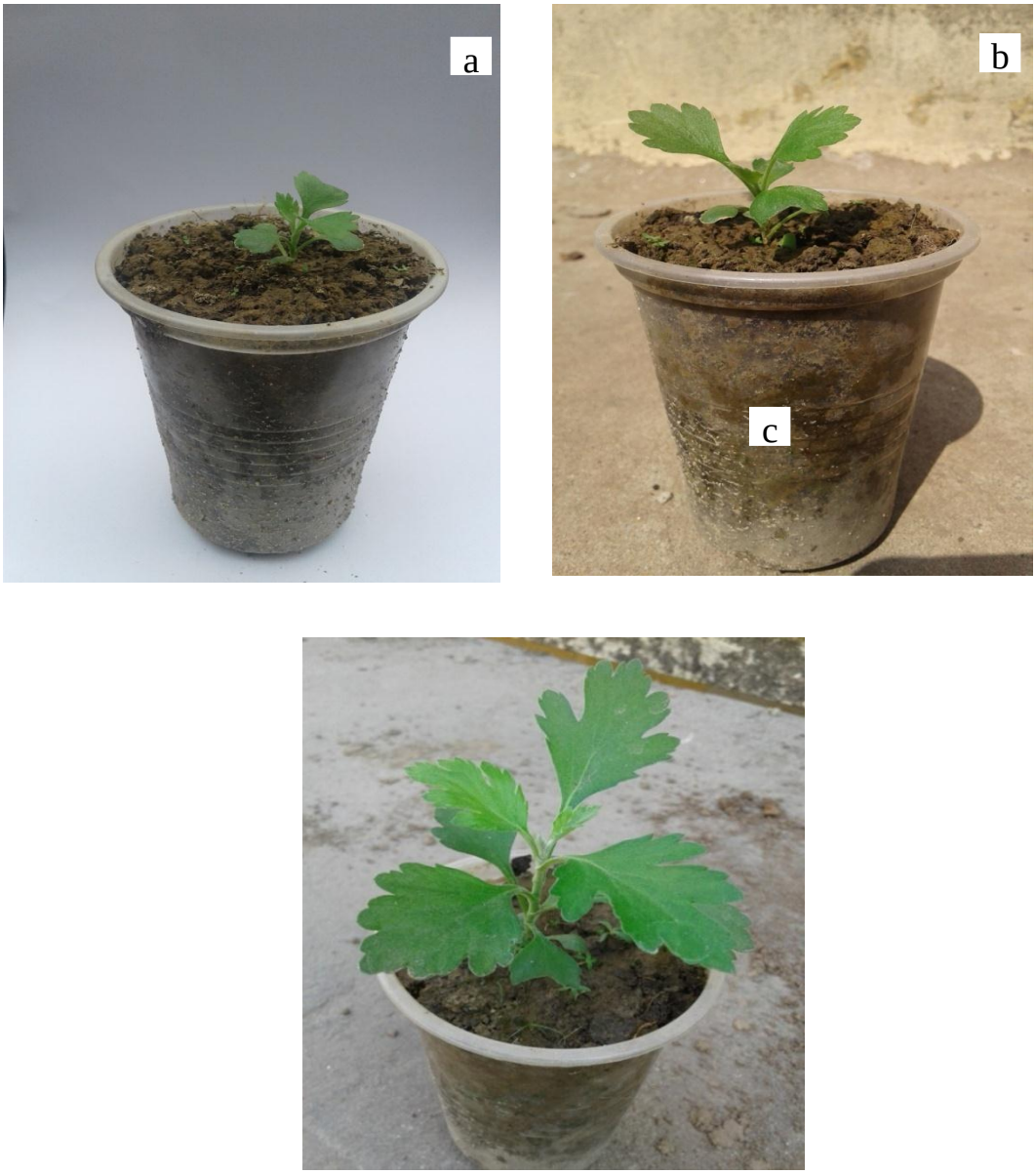

Plate 16. Hardening of chrysanthemum plantlet in shade condition (a); and in open condition (b) & (c).

CHAPTER V

SUMMARY AND CONCLUSIONS

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207 from the period of February 2016 to June 2017. The nodal segments of *Chrysanthemum morifolium* were used as experimental materials in the present investigation. Five levels of BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) were used to shoot induction. BA 2.0 mg/l with 2,4-D (0.5, 1.0, 1.5 and 2.0 mg/l), respectively were used to study callus induction potentiality. Five levels of IAA (0.5, 1.0, 1.5, 2.0 and 2.5mg/l) were used for root induction. Combined effect of BA and IAA on shoot and root induction potentiality were also investigated in chrysanthemum regeneration. The experiments were arranged in Completely Randomized Design (CRD) with 5 replications.

BA 2.0 mg/l had produced the highest frequency of shoot (80.00%) with minimum (7.60) days. BA 2.0 mg/l gave the highest number of shoot (2.20, 2.60 and 3.00) at 14, 21 and 28 DAI and the highest number of leaves (3.60, 6.20 and 9.60) at 14, 21 and 28 DAI, respectively. The highest length of shoot (1.34 cm, 2.60 cm and 3.12cm) at 14, 21 and 28 DAI, respectively was found in 3.0 mg/l BA.

Treatment BA 2.0 mg/l+ 2,4-D 1.0 mg/l had produced the highest frequency of callus (76.00%), in 14.20 days. The highest weight of callus (1.80 g, 2.70 g and 3.50 g) at 20, 30 and 40 DAI, respectively was noticed from the BA 2.0 mg/l + 2,4-D 1.0 mg/l, whereas the lowest weight of callus (0.79 g, 1.41 g and 1.90 g) at 20, 30 and 40 DAI, respectively found from BA 2.0 mg/l+ 2,4-D 2.0 mg/l. No callus was produced in control treatment. The highest number of shoot (1.80, 2.60 and 3.20) at 20, 30 and 40 DAI, respectively was obtained from the BA 2.0 mg/l+ 2,4-D 1.0 mg/l within minimum 9.20 days.

IAA 0.5 mg/l had produced the highest percentage of root initiation (80.00%) with in minimum 13.00 days. The highest number of root (2.60, 3.60 and 5.40) at 14, 21 and 28 DAI, respectively found in 1.5 mg/l IAA and the control treatment found the lowest

number of root at all DAI. The highest length of root (2.16 cm, 2.76 cm and 3.16 cm) at 14, 21 and 28 DAI, respectively) was found in 1.0 mg/l IAA.

The maximum percentage (76.00%) of shoot induction was noticed in treatment BA 2.0 mg/l+ IAA 1.0 mg/l in minimum (7.60 days) and minimum percentage (48.00%) was noticed in control hormone free medias in maximum (15.80 days). The treatment BA 2.0 mg/l+ IAA 1.0 mg/l gave the highest number of shoot (2.20, 2.80 and 3.40) and length of shoot (2.48 cm, 2.84 cm and 3.66cm) observed at 14, 21 and 28 DAI, respectively.

The minimum days (12.20) were required for root initiation in BA 2.0 mg/l+ IAA 1.0 mg/l concentration. The highest percentage (76.00%) of root induction was recorded with BA 3.0 mg/l + IAA 1.5 mg/l, whereas the lowest percentage (24.00%) of root induction was recorded in control condition. The treatment BA 2.0 mg/l + IAA 1.0 mg/l gave the highest number of root (2.40, 3.20 and 4.20) and the highest length of root (2.50 cm, 3.60 cm and 4.40 cm) at 14, 21 and 28 DAI, respectively.

So, finally it can be concluded that, a convenient protocol of rapid regeneration of chrysanthemum is established. From the above summary, the results of the present study indicated that chrysanthemum could be successfully micro propagated with 2.0 mg/l BA for rapid shoot regeneration and proliferation. BA 2.0 mg/l with 2,4-D 1.0 mg/l showed the good performance for callus and shoot induction and lower dose of IAA 0.5 mg/l alone comparatively gave the best performance in case of root. Considering the finding of the study MS medium supplemented with 2.0 mg/l BA+1.0 mg/l IAA showed the best response for shoot and root formation.

RECOMMENDATIONS

Based on the summary and conclusions following recommendations can be made:

i. For further study BAP, KIN, NAA and IBA etc. types of cytokinin and auxin can be used for more trial.
ii. Except nodal segment other explants like shoot tip, petiole, leaf and root portion could be practiced for culture.
iii. For callus induction, NAA or other callus induction hormone could be used individually or in combine dose for large number of shoot induction.
iv. Influence of other factors (elicitors, antioxidants) such as ascorbic acid, activated charcoal should be considered.
v. Further more research can be done with more variety of Chrysanthemums.

REFERENCES

Arora, J.S. (1990). Introductory Ornamental Horticulture. (Kalyani Publishers, New Delhi) 48.

Akter, N. (2001). Effect of different explants and concentrations of NAA on the callus induction and plant regeneration of brinjal cv. Uttara. M. S. thesis, Dept. of Hort., Bangladesh Agricultural University, Mymesingh. pp. 48-50.

Bajaj, Y.P.S., Springer, V., Berlin and Tliedelberg, (1991). The effect of nature and origin of explants on micropropagation. In: Biotechnology in Agriculture and Forestry. **17**: 142-167.

Bhattacharya, P., Dey, S., Das, N. and Bhattacharya, B.C. (1990). Rapid mass propagation of *Chrysanthemum morifolium* by callus derived from stem and leaf explants. Plant Cell Rep. **9**: 439-442.

Bo, C., Roest, S. and Bokelmann, G.S. (2005). Mutation breeding of *Dendranthema grandiflorm* Snow ball using *in vivo* and *in vitro* technoiques. Euphytica. **25**:11-19.

Chakarbarty, D., Mandal, A.K., and Datta, S.K. (2000). Management of chimeras through direct shoot regeneration from florets of chrysanthemum (*Chrysanthemum morifolium* Ramat). Journal of Horticultural Science. **46**: 21-34.

Chang, S. F. and Huang, M.C. (2000). The variation in tissue cultured derived plants in chrysanthemum. Journal of Chinese Society for Horticultural Science. **46**: 21-34.

Chitra, R., Arulmozhiyan, R., Jawaharlal, M., Indhumathi, K. and Vadivel, E. (2006). Rapid multiplication of chrysanthemum (*Dendranthema grandiflorum* Tzvelev) through *in vitro* culture. Journal of Ornamental Horticulture. **9**: 58- 60.

Christian, T., Kahia, J., Asiimwi, T., Mushimiyimana, I., Waweru, B., Kouassi, M., Koffi, E., Kone, S., and Sallah, P.Y. (2013). *In vitro* regeneration of pyretherum (*Chrysanthemum cinerariaefolium*) planlets from nodal explant of *in vitro* raised planlets. Inter. J. of Application or Innovation in Engineering and Management. **2**(7): 2319-4847.

Chung, H. and Park, U. (2005) *In vitro* culture of chrysanthemum. Acta Botanica Sinica. **7**(14): 90-95.

Dao, T.B., Nguyen, P.D., Do, Q.M., Vu, T.H., Le, T.L., Nguyen, T.K.L., Nguyen, H.D. and Nguyen, X.L. (2006). *In Vitro* Mutagenesis of Chrysanthemum for Breeding. Plant Mutation Report. **1**(2): 26-27.

Datta, S.K. (2008). Chrysanthemum cultivars evolved by induced mutation at National Botanical Research Institute. **49**: 72-75.

Fu Yun, L. (2010). Tissue culture and rapid propagation of chrysanthemum. Journal of Anhui Agricultural Science. **12**: 2010-24.

Gambrog, O.L., Shyluk, J.P. and Shahin, E.A. (1972). Isolation, fusion and culture of plant protoplasts. In. Plant tissue culture: methods and applications in agriculture (Ed. Thorpemta) Academic Prees, New York. pp, 115-153.

Gomez, K.A. and Gomez, A.A. 1984. Statistical ProceduresforAgricultural Research. 2nd Edn John Willey and Sons, New York, USA, ISBN: 680.

George, E.F. (1993). Plant propagation by tissue culture 2nd Edi.Part 1.The technology. Exegetics Ltd, Basingstoke, UK.

Hahn E.J., Boe J.H. and Lee Y.B. (1998). Growth and leaf surface characteristics of Chrysanthemum plantlets in micro propagation and micro ponic system. Journal of Korean Society Horticultural Science. **39**: 838-842.

Helmento, H. and Rivai, R.R. (2015). Callus Induction of Chrysanthemum indicum for increasing genetic diversity from somatic cell. Pros SemNasMasyBiodivIndon. ISSN: 2407-8050.

Himstedt, J.P., Jacobsen, H.J., Fisher-Klaver, G., Sorvani, S. and Karshu, S., Kanervo, E. and Pihakaski. (2001). Shoot regeneration from stem and leaf explants of chrysanthemum. Acta-Hort. 421-424 pp.

Hussain, I., Rashid, H.,Chaudhary, Z.,Quraishi, A.,Arjumand, S.S. and Amin.S. (1994).Effect of explant source and age of seedlings on callus formation of carnation Pakistan Journal of Agriculture Research. **15**(1): 49-53.

Hobbie, L.J. (1998). Auxin: molecular genetic approaches in Arabidopsis. Plant Physiology Biochemistry. **36**: 91-102.

Ilahi, I., Jabeen, M. and Nazneen, S.S. (2007). Rapid clonal propagation of chrysanthemum through embryogenic callus formation. Pakistan Journal of Botany. **39**(6): 1945-1952.

Jaramillo, K. (2008). Chrysanthemum cultivars evolved by induced mutation. Acta Horticulturae. **545**: 278-282.

Jeremovic, S., Nikolic, M., Subotic, A. and Radojevic, L. (2006). Clonal fidelity of chrysanthemum regenerated from long term cultures. Institute for Biological Research. **38**(3): 423-249.

Karim M.Z., Amin M.N., Azad M.A.K., Begum F., Islam M.M. and Alam R. (2002). Effect of different Plant Growth regulators on in vitro Shoot Multiplication of Chrysanthemum morifolium. Online Journal of Biological Science. **3**(6): 553-560.

Kazeroonian, N., Power, J.B. and Davey, M.R. (2014).An assessment of somaclonalvariation on Chrysanthemum cv. “Rosomee Splendid”. Scientia Horticulture. **38**: 287-294.

Keresa, S., Mihovilovic, A., Baric, M., Zidovec, Skelin, M. (2012_. The micropropagation of chrysanthemum via axillary shoot proliferation and highly efficient plant regeneration by somatic embryogenesis. African Journal of Biotechnology. **22**: 602-603.

Kim, C.K. and Naing, A. 2013.Primary and secondary somatic embryogenesis in Chrysanthemum cv. Euro. Plant Cell Tisue Organ Culture. **112**: 361–368

Kim, C.K. Lim, B.K., Chung, Y.M., Park, I., and Naing, A. (2015).Optimization of factors affecting efficient shoot regeneration in chrysanthemum cv. Shinma. Brazilian Journal of Botany. **14**: 87-90.

Labade, G.B., Dale, N.S., Umbarkar, R.B., Gadhe, S.K. and Rote, Y.N.2016. *In Vitro* Regeneration of Chrysanthemum *(Chrysanthemum morifoliumL.).* International Journal of Information Research and Review. **03**(11): 3043-3045.

Liu, S., Wang, H., Zhang, J., Evans, N., Liu, Y., Yang, W. and Guo, X. (2007). *In vitro* mutation and selection of double haploid *Brassica napus* lines with improved resistance to *Seclerotinia sclerotiorium*. Plant Cell Report. **24**: 13-14.

Lokanaha, R.D. and Maheshwaramma, S. (2009)**.** Micropropagation studies in *Chrysanthemum* with regards to hormonal concentration and type of explant. The Ashian Journal of Horticulture. **3**(1): 90-92.

Long V., Vinh B., Don N., Thuy D. and Nhut D. (2006). Microponic technology in disease free chrysanthemum production. Journal of Applied Horticulture. **7**: 67-73.

Mandal, A.K.A., Chakrabarty, D., Datta, S. (2000).*In vitro* isolation of solid novel flower colour mutants from induced chimeric ray florets of chrysanthemum. *Euphytica*. **114**: 9-12.

Mani, T. and Senthil, K. (2011). Multplication of Chrysanthemum through Somatic Embryogenesis. Ashian Journal of Pharm technology. **2**(1): 13-16.

Meena, K. Varhese, T.M., and Kumari, M. (2001). Effect of different growth regulation on fresh and dry weight of callus and regeneration in chrysanthemum cultivars universal and snow ball. Journal of Ornamental Horticulture. **6**: 188-194.

Minas, R.A. and Trigiano, R.N. (2007). Somatic embryogenesis and plant regeneration from apical meristem of *Dendranthema grandiflorum*. Journal of American Society for Horticulture Science. **116**: 366-371.

Mishra, A.K., Tripathi, N.K., Chauhan, U.K., Kumar, S. and Singh, S.K. (2006). Improved microopropgation protocol in chrysanthemum (*Dendranthema grandiflora* Tzevlev). Journal of Ornamental Horticulture. **9**: 85-90.

Murashige, T and Skoog, T. 1962. A revised medium for rapid growth and bio assays tobacoo tissue cultures. Physio.Plant. **15**: 473-497.

Murgayanti, S.E., Wieny,Rizky, H. and Rustiani, S. (2010). Callus formation and plant regeneration of chrysanthemum leaf disks explants through *in vitro*. International Seminar on Horticulture to Support Food Security. **4**: 22-23.

Nahid J S, Shyamali S and Kazumi H. 2007. High frequency shoot regeneration from petal explants of *Chrysanthemum morifolium* Ramat, *in vitro.* Pakistan Journal of Biological Sciences. **10**(19): 3356-3361.

Nalini R. 2012. Microprogation on Chryanthemum (*Chrysannthemummorifolium*) using shoot tip as explant. International Journal of Food Agriculture and Veterinary Science. **2**(2): 62-66.

Nhut, D.T., Don, N.T., An, T.T.T., Van, T.P.T., Vu, N.H., Huyen, P.X. and Khiem, D.V. (2005). Microponic and hydroponic techniques in disease free chrysanthemum (*Chrysanthemum* sp.) production. Journal for Applied Horticulture.**7**: 67-71.

Prasad, R.N., Sharma, A.K., Chaturvedi, H.C. (1983). Clonal multiplication of *Chrysanthemum morifolium* Otomezakura in long-term culture. Bangladesh Journal of Botany. **12**: 96-102.

Pierik, R.L.M. (1987). *In vitro* propagation of higher plants. Martinus Nizho of Publishers, Boston.

Rademaker, W. and De Jong, J. (1990). Genetic variation in adventitious shoot formation in *Dendranthema grandiflora* (*Chrysanthemum morifolium*) explants. In: de Jong J (ed) Integration of *In Vitro* Techniques in Ornamental Plant Breeding pp: 34-38. CPRODLO, Wageningen.

Rajalakshmi, G., Komathi, S., Raviganesh, B., Poongodi, N. and Sasikala, N. (2013).*In-vitro* micropropagation and antimicrobial activity of *Chrysanthemum indicum. Scholars* Academic Journal of Pharmacy. **4**(4): 285-288.

Rout, G.R. and Dos, P. (1997). Recent trends in the biotechnology of *Chrysanthemum*, a critical review, Scientia Horticulturae. **69**: 239-257.

Sarkar, R.H. and Shaeen, N. 2009. *In vitro* propagation of chrysanthemum (*Chrysanthemum morifoliumn* Ramat) through callus culture. Plant Tissue Culure. **11**(1): 85-91.

Sarkar, R.H. and Shaeen, N. (2009). *In vitro* propagation of chrysanthemum (*Chrysanthemum morifolium* Ramat) through callus culture. Plant Tissue Culure.**11**(1): 85-91.

Sauvaire, D. and Galgy, R. (1978). Multiplication of vegetative dela Cannal a sourse par bounturage *in vitro.* CRACad D Science Se. **287**: 446-470

Schenk, R. and Hiberlandt, G. (1976). Cultrversuchenitisolacten. Pilansenzelen Sitz Ber Mal Nat ki Kais Adad Wiss Wien.**111**: 62-72.

Sen, J., Kalia, S. and Mukherjee, S.G. (2002). Level of endogenous free amino acid during various stages of culture of Vigna mungo (L) somatic embryogenesis, organogenesis and plant regeneration. Cun: Science. **82**(4): 429-433.

Seo, S.Y., Sang, Y., Choi, D.C., Kim, J.M., Lim, H.C., Kim, H.J., Choi, J.I., Choi, Y.G., Lee, J.M. and Zhang, D.L. (2003). Plant regeneration from leaf explants and efficient Ago-bacterium mediated transformation system of chrysanthemum. Acta Horticultrae. **336**: 333-338pp.

Shatnawi M.A. (2006). Micropropagation and germplasm storage of *Prunus amygdalus* by the vitrification method. Jordan Journal of Agricultural Science. **2**(3): 222-233.

Soo, C.C. (2014). Influence of media on *in vitro* root regeneration and micropropagation of *Chrysanthemum morifolium* Ramat cv. Hwiparam. Journal of Life Science. **11**(9): 340-720.

Sun, M., Davey M.R. and Power, J.B. (2007). *In vitro* propagation of chrysanthemum CV. Cheng Yun through callus culture. Plant Tissue Culture. **11**(1): 56-62.

Teixeira da Silva (2003).Chrysanthemum organogenesis through thin cell layer technology and plant growth regulator control.Asian journal of plant science. **2** (6): 505-514.

Tiller, R. (2014). Microprogation on Chryanthemum cv. kulo using nodules as explant. International Journal of Food Agriculture and Veterinary Science. **2**(2): 56-62.

Trifuncovic, P., Cholsez, G.D. and Dekeyser, A. (2006). Application of *in vitro* culture to vegetative propagation of chrysanthemum cv. 'White Spider'. Blletindela Societe Royale de Botanique de Belgique. **116**: 161-166.

Verma, O.P. (2012). Standerdisation of auxin concentration for root induction in *Chrysanthemum morifolium.* Advances in Applied Science Research. **3**(3): 1449-1453.

Wankhede, K.N.,Narkhede, M.N., Shivankar, R.S. and Rathore, T.H. (2000). Callus induction and micropropagation studies in chrysanthemum. Annuals of Plant Physiology. **14**: 174-177.

Waseem, K., Jilani, M.S. and Khan, M.S. (2009). Rapid plant regeneration of chrysanthemum (*Chrysanthemum morifolium* I.) through shoot tip culture. African Journal of Biotechnology. **8**(9): 1871-1877.

Waseem, K., Saleem, J.M., Khan, M.S., Kiran, M, and Khan, G. (2011).Efficient *in vitro* regeneration of chrysanthemum (*Chrysanthemum morifolium L.*) plantlets from nodal segments. African Journal of Biotechnology. **10**(8): 1477-1484.

Zalewska, M. and Tymoszuk, A. (2014). Biological factors affecting regeneration of adventitious shoots from *in vitro* isolated ligulate florets of chrysanthemum. Acta Sci. Pol., HortorumCultus. **13**(3): 155-165.

Zalewska, M., Miler, N., Wenda, P. (2010). Effect of *in vitro* topophysis on the growth, development, and rooting of chrysanthemum explants. Journal of Horticultural Science & Biotechnology. **85**(4): 362–366.

APPENDICES

Appendix I. Analysis of variance (ANOVA) of effect of different concentration of BA on days to shoot initiation, percent of shoot and number of shoot at different DAI

Source of variance	d.f.	Days to shoot induction	Percent (%) Shoot initiation	Number of Shoot		
				14 DAI	21 DAI	28 DAI
Treatments	5	71.600	981.333	0.693**	0.913**	1.313**
Error	24	0.383	120.00	0.250	0.267	0.283
Total	29					

**= Significant at 1% level of Probability.

Appendix II. Analysis of variance (ANOVA) of effect of different concentration of BA on length of shoot at different DAI

Source of variance	d.f.	Length of Shoot (cm)		
		14 DAI	21 DAI	28 DAI
Treatments	5	0.575**	1.729**	0.871**
Error	24	0.002	0.004	0.015
Total	29			

**= Significant at 1% level of Probability.

Appendix III. Analysis of variance (ANOVA) of effect of different concentration of BA on number of leaves at different DAI

Source of variance	d.f.	Number of leaves		
		14 DAI	21 DAI	28 DAI
Treatments	5	0.773**	3.013**	10.353**
Error	24	0.150	0.167	0.267
Total	29			

**= Significant at 1% level of Probability.

Appendix IV. Analysis of variance (ANOVA) of effect of BA and 2,4-D on days to callus initiation, percent of callus and weight of callus at different DAI

Source of variance	d.f.	Days to callus initiation	Percent of Callus	Weight of callus		
				20 DAI	30 DAI	40 DAI
Treatments	4	310.200**	5224.000	2.448**	5.227**	8.505**
Error	20	0.760	5224.000	0.001	0.004	0.007
Total	24					

**= Significant at 1% level of Probability.

Appendix V. Analysis of variance (ANOVA) of effect of BA and 2,4-D on days to shoot initiation, and number of shoot at different DAI

Source of variance	d.f.	Days to shoot initiation	Number of shoot		
			20 DAI	30 DAI	40 DAI
Treatments	4	212.500**	2.660**	5.540**	7.760**
Error	20	1.200	0.0220	0.220	0.180
Total	24				

**= Significant at 1% level of Probability.

Appendix VI. Analysis of variance (ANOVA) of effect of different concentration of IAA on days to root initiation, percent of root and number of root at different DAI

Source of variance	d.f.	Days to root initiation	Percent of root initiation	Number of root		
				14 DAI	21 DAI	28 DAI
Treatments	5	128.213**	67.267**	1.473**	2.780**	8.853**
Error	24	1.033	13.432	0.150	0.167	0.233
Total	29					

**= Significant at 1% level of Probability.

Appendix VII. Analysis of variance (ANOVA) of effect of different concentration of IAA on length of root at different DAI

Source of variance	d.f.	Length of root (cm)		
		14 DAI	21 DAI	28 DAI
Treatments	5	2.210**	2.814**	2.950**
Error	24	0.007	0.011	0.012
Total	29			

**= Significant at 1% level of Probability.

Appendix VIII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IAA on days to shoot initiation, % of shoot initiation and number of shoot at different DAI

Source of variance	d.f.	Days to shoot initiation	Percent of shoot initiation	Number of Shoot		
				14 DAI	21 DAI	28 DAI
Treatments	25	21.788**	425.477 **	0.288**	0.821**	1.401 **
Error	104	0.850	140.000	0.077	0.331	0.350
Total	129					

**= Significant at 1% level of Probability.

Appendix IX. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IAA on length of shoot at different DAI

Source of variance	d.f.	Length of shoot (cm)		
		14 DAI	21 DAI	28 DAI
Treatments	25	1.986**	1.978**	2.552**
Error	104	0.020	0.016	0.012
Total	129			

**= Significant at 1% level of Probability.

Appendix X. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IAA on number of leaves at different DAI

Source of variance	d.f.	Number of leaves		
		14 DAI	21 DAI	28 DAI
Treatments	25	7.520**	17.865**	22.12**
Error	104	0.145	0.221	0.734
Total	129			

**= Significant at 1% level of Probability.

Appendix XI. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IAA on days to root initiation, percent of root and number of roots at different DAI

Source of variance	d.f	Days to root initiation	% Root initiation	Number of root		
				14 DAI	21 DAI	28 DAI
Treatments	25	84.229**	593.231**	0.812**	1.468**	3.435**
Error	104	1.031	43.077	0.069	0.096	0.138
Total	129					

**= Significant at 1% level of Probability.

Appendix XII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IAA on length of root at different DAI

Source of variance	d.f.	Length of root		
		14 DAI	21 DAI	28 DAI
Treatments	25	2.115**	3.853**	5.868**
Error	104	0.020	0.026	0.263
Total	129			

**= Significant at 1% level of Probability.