IN VITRO REGENERATION OF *Caladium bicolor*

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DHAKA-1207

JUNE, 2014

IN VITRO REGENERATION OF *Caladium bicolor*

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REGISTRATION NO. 07-02476

A Thesis
Submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

SEMESTER: JANUARY-JUNE, 2014

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CERTIFICATE

This is to certify that thesis entitled, ***"IN VITRO* REGENERATION OF *Caladium bicolor"*** *submitted to the Faculty of* **AGRICULTURE,** *Sher-e-Bangla Agricultural University, Dhaka, Bangladesh, 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* **KHANDAKER SOHAEL AHMED**, *Registration No.* **07-02476** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

Date: June, 2014
Place: Dhaka, Bangladesh

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LIST OF ABBREVIATED TERMS

Abbreviation		Full Word
Agril.	:	Agricultural
Biol.	:	Biological
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
Kin		Kinetin
BARI	:	Bangladesh Agricultural Research Institute
cm	:	Centimeter
CRD	:	Completely Randomized Design
cv.	:	Cultivar
Conc.	:	Concentration
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
WAI	:	Weeks After Induction
Dw	:	Distilled water
DMRT	:	Duncan's Multiple Range Test
et. al.	:	And others
FAO	:	Food and Agriculture Organization
g/L	:	Gram per litre
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
NAA	:	α-Napthalene acetic acid

Int.	:	International
J.	:	Journal
Mol.	:	Moleculer
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Etcetera

ACKNOWLEDGEMENT

At first I express my best gratitude to Almightly Allah for his never-ending blessing to complete this work successfully. It is a great pleasure to express profound gratitude to my respected parents, who entiled much hardship inspiring for prosecuting my studies, thereby receiving proper education.

I would like to to express my earnest respect, sincere appreciation and enormous thankfulness to my reverend supervisor, **Prof. Dr. Ekramul Hoque**, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, for hir scholastic supervision, continuous encouragement and constructive suggestion and unvarying inspiration throughout the research work and for taking immense care in preparing this manuscript.

I wish to express my gratitude and best regards to my respected Co-Supervisor, **Dr. Md. Aziz Zilani Chowdhury**, Chief Scientific Officer, Bangladesh Agricultural Research Council Dhaka, for his continuous direction, constructive criticism, encouragement and valuable suggestions in carrying out the research work and preparation of this thesis.

I am highly grateful to my honorable teacher **Homayra Huq,** Chairman, Department of Biotechnology, Sher-e-Bangla Agricultural University, for his valuable teaching, direct and indirect advice, and encouragement and cooperation during the whole study period.

I am also highly grateful to my honorable teachers Mohammad Nazrul Islam, Md. Abdul Halim, Department Biotechnology, Sher-e-Bangla Agricultural University, for their valuable teaching, direct and indirect advice, encouragement and cooperation during preparation of this thesis.

I am also thankful to my honorable teacher Prof. Dr. A.F.M. Jamal Uddin Department of Horticulture for giving me valuable advice and direction during research materials collection and thesis paper preparation.

I am also grateful to my honorable teacher Prof. Dr. Md Razzab Ali, Department of Entomology, Sher-e-Bangla Agricultural University, for giving me valuable suggestions during data analysis and thesis paper preparation.

I feel proud of expressing my sincere appreciation and gratitude to Minister of Science and Technology of People's Republic of Bangladesh for selecting me National Science and Technology (NST) fellow and funding.

I feel to expresses my heartfelt thanks to all the teachers and staff of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka for their valuable suggestions and encouragement during the period of the study.

I would like to thank all my friends and well aishers who always inspired me during my research specially Md Arifur Rahman who helped me with his valuable suggestions and directions during the preparation of this thesis paper.

I can never repay the debt of my beloved father **Alhaj Md. Maqbul Hussain**, mother **Alhaj Most. Shapna Begam**, uncle, aunty, sisters, brothers and well wishers for their inspiration, constant encouragement and sacrifice for my higher education specially my elder brother **Dr. Khandakar Shamim Ahmed**, Associate Professor, Department of Bangla, Jahangirngar University, Dhaka whose inspiration guiding me toward the achievement of my goal.

I expresses my immense gratefulness to all of them who assisted and inspired me to achieve higher education and regret for my inability for not to mention every one by name.

The Author

IN VITRO REGENERATION OF *Caladium bicolor*

ABSTRACT

The experiment was conducted at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh during the period from October 2013 to July 2014 to investigate the effects of BA, Kin, IBA, IAA, NAA for *in vitro* regeneration of *Caladium bicolor using* shoot tip explants. The work was designed in CRD with three replications. Maximum number of shoot (5) initiated when MS media was supplemented with 1.0 mg/L BA and minimum (1) was at control. Highest percentage (90%) of explants showed shoot induction at BA 0.50 mg/L +2.00 mg/L IBA and minimum shoot induction days (40.47) were recorded at BA 1.00 mg/L +2.00 mg/L IBA. Highest number of shoots (19.67) were recorded at BA 1.00 mg/L +2.00 mg/L IBA. Longest length of shoot (11.43 cm) was recorded at BA 0.25 mg/L +2.50 mg/L IBA. Maximum number of leaves were recorded at BA 1.00 mg/L +2.00 mg/L IBA. To investigate root formation potentiality and root morphology NAA, IAA and IBA at different concentrations were used. Among the rooting hormones IAA at 2.0 mg/L concentration showed maximum percentage (90%) of root induction. Maximum numbers (29.87) of root were recorded when IAA was used at 2.0 mg/L concentration. Highest length of root (10.20 cm) was recorded at 2.0 mg/L IAA. Ultimately combined effect of BA and IBA seemed better than individuals based on average performance of growth parameters. Survival rate was 92% in open field condition. Reliable protocols for micropropagation of *Caladium bicolor* were established and could be used for large scale production of disease free, high yielding and premium quality planting material.

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CHAPTER I

INTRODUCTION

Caladium species commonly known as “Angels wing” belongs to family Araceae. Plants of the Family Araceae are widely cultivated as ornamentals and for food. The genus *Caladium* has seven prominent species, which are all tropical and originated from the Amazon Basin in areas such of South America. There are around 100 different cultivars available in the commercial market with only about 20 making up the bulk of production (Anonymous, 2004).

Caladium is one of the commercially important ornamental crops. It occupies a very selective and special position to the people because of its prettiness, elegance and sweet pleasant color harmony. Ornamental value of *Caladiums* depends to a great extent on leaf characteristics, including shape, colour, colour pattern, and venation pattern (Deng and Harbaugh, 2005). The ornamental value of caladiums used as pot or landscape plants is determined primarily by leaf characteristics. Improving leaf characteristics or generating new combinations of them has been one of the most important objectives in caladium breeding and cultivar development (Wilfret, 1993).

Caladium (*Caladium bicolor Vent*.) are important floriculture industry of South America where 95% of the world’s caladium grows. From here they are shipped nationwide and across making an important source of income. Caladiums are excellent landscape and pot plants (Deng and Harbaugh, 2006) grown for their colorful leaves that have a combination of green and white, green and red, white with red blotches or green veins and some have lavender spots. The size of the heart-shaped leaves may vary from 6 inches to 2 feet in length. The plant needs bright light but no direct sunlight. The plants are very cold sensitive and prefer temperatures above 25ºC and ample of moisture.

Caladium is generally propagated from tubers for commercial purpose but tuber propagation has limitations as tubers produce healthier plants for one season only and second year foliage is usually not as good as the first year. Therefore, more satisfactory results may be obtained by starting with new tubers each year. Commercial propagation is difficult, being seeds very small, very high mortality and plants grown from seeds are very expensive. It has also been reported that seed propagation results in variability (Gill *et al.*, 1994). Concerns have been raised about possible loss of genetic diversity due to a drastic decline in the number of cultivars in the last century (Deng *et al.*, 2007). Moreover, this method is very difficult to keep plant true to type and pathogen free (Siddiqui *et al.*, 1993).

Tissue culture has emerged as the predominant method of propagating ornamental plants and has been used to rapidly propagate disease-free *Caladium* (Hartman, 1974). *In vitro* techniques are powerful tools for plant breeders in improving the performance of agriculture, horticulture and floriculture plant species. Interest in tissue culture propagation of *Caladium bicolor* has evolved due to its ornamental importance throughout the world. Propagation through meristem has been identified as mechanism of rapid regeneration in Caladium. Micropropagation of *C. bicolor* was for the first time reported by Hartman (1974) and later on a new Caladium cultivar was discovered from *C. humboldtii* named as “Marcel” by Lecouffe (1981) through somaclonal variation (Larkin *et al.*, 1981).

Ornamental plant product plays an important role in the world trade. However cost of tuber is an important constraint in Caladium production. This production of cost can be reduced by the application of tissue culture technique and thereby Caladium productivity can be increased. Plant tissue culture now has direct commercial applications as well as value in basic research. In addition to that, it will give disease free healthy seedling for higher yield. The Caladium growers will

be benefited by the use of tissue culture regenerated material for caladium production. This produced caladium we can export as well as fulfill the domestic demand. Present investigation was undertaken to achieve large-scale multiplication and faster development of caladium through tissue culture technique using shoot tips as explant. Therefore, the present study was undertaken with the following objectives:

Objectives

1. Rapid and easy *in vitro* propagation protocol development in *Caladium bicolor;*
2. Identification of suitable hormonal combination for *in vitro* regeneration of *Caladium bicolor;*
3. Large number of plantlet production for commercial cultivation.

CHAPTER II

REVIEW OF LITERATURE

2.1. Concept of Plant tissue culture

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells, a concept proposed by Haberlandt (1902) and unequivocally demonstrated, for the first time, by Steward et al. (1958). Tissue culture and plant regeneration are an integral part of most of plant transformation strategies which is now widely used for improvement of different crops. Practically any plant transformation experiment relies on the ability to regenerate plants from isolated cells or tissues *in vitro* or using tissue culture techniques (Barcelo *et al*., 2001)

Experiments with tissue culture began in the nineteenth century when two German biologists, Shleiden and Shwann, reported that the whole plant can be reconstituted whenever cells from some plants were removed (Bonga and Aderkas, 1992). This experiment led to the concept of totipotency, suggesting that each cell is a unit capable of originating a new organism, and that each cell from a multicellular organism retains the information present in the fertilized ovule. Totipotency stimulates the regeneration of plants with small tissue mass and isolated cells and consequently, undetermined plant cells may show totipotency, plus a high degree of plasticity to physical and environmental stimulus (Cirino and Riede, 1999).

Plant regeneration *in vitro* is an alluring alternative for mass multiplication of outstanding cultivars at faster rates than conventional methods. *In vitro* culture promotes accelerated multiplication of superior clones and is a pre-requisite for the improvement of plants via genetic engineering technique. Tissue culture has been exploited to generate genetic variability by producing haploids, somaclonal and gametoclonal variants from which crop plants can be improved. It is also

utilized for enriching the health status of the plant material and to amplify the number of desirable germplasm available to the plant breeder. Tissue culture, in union with molecular tools and techniques, has been successfully used to integrate specific traits through genetic transformation. The culture of single cell and meristems can be effectively used to clean out the pathogens from planting material and thereby dramatically improve the produce of established cultivars. By using certain laboratory materials, typically taking one or two decades to attain the trade market all the way through plant breeding, this technology can be expected to have an ever accelarating impact on crop improvement as we pass through the new millennium (Kothari *et al.*, 2010).

2.2. Concept of Plant tissue culture in Ornamental plant

Burnett (1984), became the first author to bring a very rare and beautiful aroid, *Alocasia guttata var. imperialis* N.E. Brown, from the brink of obscurity back into the horticultural limelight by using a plant tissue culture technique known as micropropagation. He then made propagules available to the collectors around the world.

Tissue culture system in ornamental flowers like roses has been established (Hsia and Korban, 1996; Kintzios *et al.,* 1999; Ibrahim and Debergh, 2001; Rout *et al.,* 2006; Hameed *et al.,* 2006; Drefahl *et al.,* 2007; Previati *et al.,* 2008). Recently, *in vitro* flower induction in roses was demonstrated (Wang *et al.,* 2002; Vu *et al.,* 2006). Tissue culture techniques are applied for micropropagation and production of pathogen-free plants. (Kaviani *et al.,* 2011). Many commercial ornamental plants are being propagated by *in vitro* culture on the culture medium containing auxins and cytokinins (Preil, 2003; Rout and Jain, 2004).

The first report on shoot multiplication and rooting of rose (*Rosa multiflora*) was made by Elliott (1970) by using shoot tip explants and later on followed by others (Hasegawa, 1979; Rout et al., 1989). The techniques of stimulating axillary

branching or culturing nodal sections *in vitro* are probably most commonly used in micropropagation in chrysanthemum (Lawrence, 1981).

2.3. Explant

The success of tissue culture is related to the correct choice of explants. Shoot or shoot tips and node cultures are the most commonly used culture types in micropropagation of plants. Explants from shoot tips and nodal stem segments are suitable for enhanced axillary branching.

Hartman (1974) was the first to report the use of micropropagation for the purpose of producing disease free aroids. He carefully dissected out the shoot tips, which consisted of the apical meristem and a leaf primordium, and subsequently cultured them on a chemically defined medium. He was able to produce *Caladium bicolor*, *Xanthosoma sagittifolium* and *Colocasia esculenta* plants free of dasheen mosaic virus.

Propagation through meristem has been identified as mechanism of rapid regeneration in Caladium and the plants produced through this technology provide much export potential as they are shipped internationally with limited quarantine restrictions and it has the prospective for developing new cultivars of the species (Hyndman, 1987).

Direct or indirect *in vitro* organogenesis of the plant tissue is mostly governed by the suitable selection of the explant. In support to this statement, *Anthurium* regeneration is also reliant on the age and type of the explant concerned. Different explants including explants such as *in vitro* seedling, axillary bud, shoot tip, node, leaf, lamina, petiole, spathe and spadix (Pierik *et al*., 1974; Leffringen and Soede, 1979; Kunisaki, 1980; Kuehnle and Sugii, 1991; Teng, 1997; Joseph *et al*., 2003; Vargas *et al*., 2004; De Lima *et al*., 2006) initiate the purpose efficiently in *Anthurium* tissue culture**.**

For direct organogenesis into multiple shoot and root regeneration, shoot tip explants were successfully used (Somaya *et al*., 1998; Lara *et al*., 2004; Dhananjaya and Sulladhmath, 2006).

Nodal explant was recognized to be the other most responsive explant to regenerate *in vitro* roots (Rivero-Bautista *et al*., 2005). Comparable results have been demonstrated by other authors using micro-cuttings of *in vitro* seedlings explants (Vargas *et al*., 2004; Maira *et al*., 2009). Yang *et al*. (2002) and Martin *et al*. (2003) fascinatingly studied the efficiency of direct shoot regeneration from lamina explants.

Ali *et al.* (2007) did micropropagation of *Caladium bicolor* taking meristem (0.5-1.0) as a explants.

Sakper *et al.* (2007). Used corm, petiole and leaf as a explants during micropropagation of *Caladium humboldtii Schott.*

In vitro propagation through meristem culture is the best possible means of virus elimination and produces a large numbers of plants in a short span of time. It is a powerful tool for large-scale propagation of horticultural crops including pot plants. The term ‘meristem culture’ specifically means that a meristem with no leaf primordia or at most 1–2 leaf primordial which areexcised and cultured. The first significant use of plant tissue culture in ornamental was made during 1920s when orchid seeds were germinated under laboratory conditions (Knudson,1922).

Mayer (1956) succeeded first time regeneration of Cyclamen shoots from tuber segments on MS medium supplemented with 10.7 µM NAA.

Martin *et al.* (2003) succeeded in direct shoot bud regeneration from lamina explants of *Anthurium andraeanum* on MS medium fortified with 1.11 µM BA, 1.14 µM IAA and 0.46 µM KIN. Atta-Alla *et al*. (1998) reported the shoot bud

regeneration from leaf and petiole explants of *Anthurium parvispathum* and subsequently establishment in soil.

CHAPTER III

MATERIALS AND METHODS

3.1. Time and Location of the experiment

The present study was planned during October, 2013 to July, 2014 at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e Bangla Nagar, Dhaka-1207, Bangladesh. Materials and methods followed to conduct the present study have been presented in this chapter.

3.2. Experimental materials

3.2.1. Plant materials

Caladium bicolor were used as experimental materials in the present investigation.

3.2.2. Source of materials

The planting materials of *Caladium bicolor* were collected from different Nursery around Sher-e-Bangla Nager, Dhaka-1207.

3.2.3. Types of explants

The healthy, disease free shoot tip of pot grown caladium of 0.5-1cm length were used as explants for the study for *in vitro* regeneration.

3.2.4. 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 (Composition of MS media have been shown in appendix I). 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 ℃ temperature.

3.3. Hormone stock solution

The first step in the preparation of the medium was the preparation of hormone stock solutions. To expedite the preparation of the medium separate stock solutions for growth regulators were prepared and used.

Separate stock solution of hormones was prepared by dissolving the desired quantity of ingredients to the appropriate solvent and made the final volume with distilled water and stored in a refrigerator at 4^0C for later use.

The following growth regulators and concentrations were used in this present investigation.

Auxins

Indole butyric acid (IBA) (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L)

Indole acetic acid (IAA) (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L)

α-Napthalene acetic acid (NAA) (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L)

Cytokinins

Benzyladenine (BA) (0.25, 0.50, 0.75 and 1.0 mg/L)

Kinetin (Kin) (0.25, 0.50, 0.75 and 1.0 mg/L)

These hormonal supplements were dissolved in proper solvent as shown against each of them.

Hormones (Solute)	Solvent
BA	0.1 N NaOH
Kin	0.1 N NaOH
IBA	99% ethyl alcohol
IAA	99% ethyl alcohol
NAA	99% ethyl alcohol

To prepare the stock solution of hormones (1mg/ml), 10 mg of solid hormone was placed in a small beaker and then dissolved in 10 ml of 99% ethyl alcohol and 0.1 (N) NaOH solvent. Finally the volume was made upto 100 ml by the addition of sterile distilled water using a measuring cylinder. The prepared hormone solution was then labeled and stored at 4 ± 1^{0}C for use upto two month. (MS media manufacturing company, Duchefa Biochemie, Netherland).

3.4. MS media preparation

To prepare 1000 ml of culture media the following steps were followed:

Step-1. 700 ml of sterile distilled water was poured into 1000 ml beaker.

Step-2. 5 g of ready MS and 30 g of sucrose was added and gently stirrered to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

Step-3. Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

Step-4. The volume was made up to 1000 ml with addition of sterile distilled water.

Step-5. The pH was adjusted at 5.8.

Step-6. Finally, 8 g agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

3.5. Sterilization

For *in vitro* techniques, aseptic condition is a prerequisite. So, all instruments, glassware and culture media were sterilized.

3.5.1. Sterilization of culture medium

The culture vessels containing the medium were autoclaved with 1.06 kg/cm^2 (15 PSI) of pressure at 121^{0}C for 20 minutes. After autoclaving the culture vessels (vials) containing the medium were allowed to cool in culture racks. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explants.

3.5.2. Sterilization of glassware and instruments

Beakers, test tubes, conical flasks, pipettes, metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^{0}C for 35 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.5.3. 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.

3.6. Precaution to ensure aseptic condition

The cabinet was usually started half an hour before use and wiped with 70% ethylalcohol 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% ethylalcohol followed by flaming and cooling. Hands were also sterilized by wiping with 70% ethylalcohol. Aseptic conditions were followed during each and every operation to avoid the contamination of culture.

3.7. Explants preparation and culture

3.7.1.1. Preparation of explants

The shoot tip was the starting material. It was obtained from developing bulbs (about three months of age) of *Caladium bicolor* grown under field conditions and was brought to the preparation room (Plate 1). The bulbs were washed thoroughly under running tap water. The roots and outer tissues of the bulbs were removed with the help of a sharp knife. A number of outer scales were removed until the shoot measured about 2 to 3cm length and 2.0 cm width at the base.

Plate 1. *Caladium bicolor* plantlets of 2-3 months aged collected from nursery

3.7.1.2. Surface sterilization of explants

The shoot tip of 2 to 3 cm size was taken in a beaker (Plate 2). Surface sterilization of explants was done as follows:

i. The bulbs were cut as small size (2 to 3 cm) and rinsed with running tap water.
ii. The shoot tips were soaked with Tween-20 solution having 10% concentration for 15 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.

(iv-viii no. activities were done in Laminar air flow cabinet)

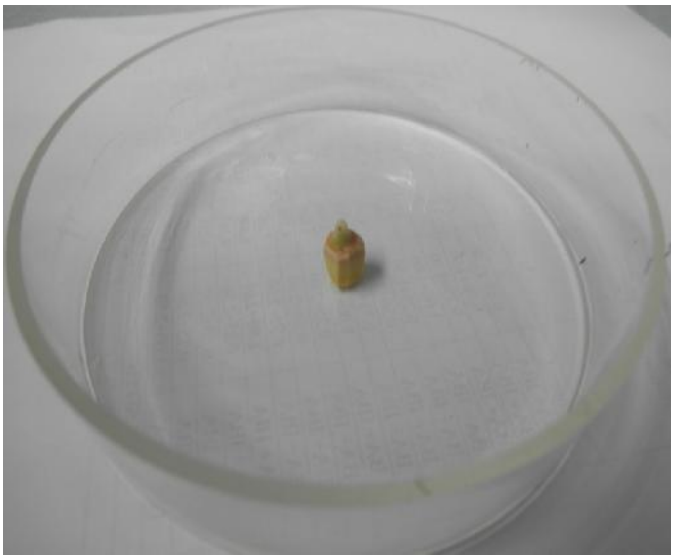

Plate 2. *Caladium bicolor* explant (shoot tip) prepared for placement in MS media

3.7.2. Culture of explants

3.7.2.1. Inoculation of culture

The isolated and surface sterilized shoot tip was collected carefully through maintaining aseptic condition inside the laminar air flow cabinet. The individual shoot tip were directly inoculated to each of the culture tube containing 15 mL of MS medium supplemented with different concentrations of hormones as per treatment.

3.7.2.2. Incubation

The culture vials transferred to culture racks and allowed to grow in controlled environment. The temperature of the growth room was maintained within 25 ± 1^{o}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.

3.7.2.3. Maintenance of proliferating shoots

Initial sub-culturing was done after 40 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 20-25 days.

3.7.2.4. Regeneration of plants from *in vitro* proliferated buds

In vitro proliferated micro shoots were separated and each of the micro shoot was placed on culture medium, which was supplemented with particular concentration of hormone for shoot differentiation.

3.7.2.5. Root induction of regenerated shoots

When the shoots grew about 2-4 cm in length with 2-6 well developed leaves they were removed aseptically from the culture tubes and were separated from each other and again cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction.

3.8. Acclimatization of the regenerated plantlets

Regenerated plantlets were transplanted to pots (10×15cm) containing soil and cowdung in 1:1 ratio and soil mixture were treated with a solution of 1% IBA. Occasional spray of water was done to prevent sudden desiccations and maintain high humidity around the plantlets. Initially the plantlets were hardened in growth chamber. Then after 2 weeks, exposed to lower humidity and higher light intensity. Finally, after 20 days plantlets were transferred to natural environment.

3.9. Treatments

Four experiments were conducted to assess the effect of different concentrations of BA and IBA on shoot proliferation and subsequent rooting of the multiplied shoot.

Sub-experiment 1. Effect of BA and Kin on shoot induction potentiality in *Caladium bicolor*

In this experiment, bulbs of *C. bicolor* were used as sources of shoot tip to investigate the effect of BA and KIN.

Treatments: Four levels of BA & KIN (0.25, 0.50, 0.75 and 1.0 mg/L) and a control (0.0 mg/L) were used. The experiments were arranged in Completely Randomized Design (CRD) with 3 replications. Each of replications consisted of 10 culture vials.

Sub-experiment 2. Combined effect of BA and IBA on shoot induction potentiality shoot morphology in *Caladium bicolor*

In this sub-experiment 16 combinations of BA and IBA were practiced. The combinations were as follows

Treatments: 16 combinations of BA and IBA were made. Combinations were used as follows

T_2 = BA 0.25 mg/L +1.00 mg/L IBA

T_3 = BA 0.50 mg/L +1.00 mg/L IBA

T_4 = BA 0.75 mg/L +1.00 mg/L IBA

T_5 = BA 1.00 mg/L +1.00 mg/L IBA

T_6 = BA 0.25 mg/L +1.50 mg/L IBA

T_7 = BA 0.50 mg/L +1.50 mg/L IBA

T_8 = BA 0.75 mg/L +1.50 mg/L IBA

T_9 = BA 1.00 mg/L +1.50 mg/L IBA

T_{10} = BA 0.25 mg/L +2.00 mg/L IBA

T_{11} = BA 0.50 mg/L +2.00 mg/L IBA

T_{12} = BA 0.75 mg/L +2.00 mg/L IBA

T_{13} = BA 1.00 mg/L +2.00 mg/L IBA

T_{14} = BA 0.25 mg/L +2.50 mg/L IBA

T_{15} = BA 0.50 mg/L +2.50 mg/L IBA

T_{16} = BA 0.75 mg/L +2.50 mg/L IBA

T_{17} = BA 1.00 mg/L +1.00 mg/L IBA

And T_1 = control (0.0 mg/L)

The experiments were arranged in Completely Randomized Design (CRD) with 3 replications. Each of replications consisted of 10 culture vials.

Sub-experiment 3. Effect of NAA, IAA and IBA on root formation potentiality and root morphology of micro propagated shoots in *Caladium bicolor*

Four levels of NAA, IAA and IBA (1.0, 1.5, 2.0 and 2.5 mg/L) and control (0.0 mg/L) were used as treatments. The experiments were arranged in Completely Randomized Design (CRD) with 3 replications. Each of replications consisted of 10 culture vials.

Sub-experiment 4. *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.

3.10. Data collection

Data were collected on the effect of different treatments on shoot parameters recorded at 3, 5, and 8 weeks after induction (WAI) and root proliferation on different treatments recorded at 3, 5 and 8 weeks after induction (WAI).

3.10.1. Regeneration potentiality (%)

Regeneration potentiality was calculated by percentage of explants responded using the following formula.

$$\text{Percentage of explants responded} = \frac{\text{Number of explant induced shoot}}{\text{Total number of explants inoculated}} \times 100$$

3.10.2. Days to shoot induction

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

3.10.3. Number of shoots per explant

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

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

3.10.4. Shoot length (cm)

Shoot length was measured in centimeter (cm) from the base to the top of the explants by a measuring scale. The mean was calculated.

3.10.5. Number of leaf

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

3.10.6. Number of roots

The number of roots per plantlet was counted and the mean was calculated.

3.10.7. Length of root (cm)

Root length was measured in centimeter from the base to the tip of the roots and the mean was calculated.

3.10.8. 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:

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

3.11. Statistical analysis of data

All the experiments regarding shoot, root induction and plantlet regeneration were done under laboratory condition following Completely Randomized Design (CRD). In all experiments, each treatment was replicated three times and ten explants were included in each replication. The collected data on different parameters were analyzed using a MSTAT-C package computer program. The analysis of variance was performed and mean were compared by Least Significant Difference (LSD) test for interpretation of results.

CHAPTER IV

RESULTS AND DISCUSSION

The experiment was conducted to investigate the effect of different phytohormones in subsequent shoot and root regeneration in *Caladium bicolor*. The following works were conducted at the Biotechnology lab, Department of

Biotechnology, Sher-e-Bangla Agricultural University. The results of the experiment were presented and discussed in this chapter with Plates (3-11), Figures (1-4) and Tables (1-6). Analyses of variance in respect of all the parameters have been presented in Appendices I-XIX.

4.1. Sub-experiment 1. Effect of BA and Kin on shoot induction potentiality in *Caladium bicolor*

Plant regeneration and subsequent shoot and root multiplication from shoot tip of *Caladium bicolor* was done. MS medium supplemented with different concentrations of BA and KIN were used and healthy shoot tips were collected and cut into pieces. Each piece having 1-2 cm long and about 1cm diameter was used as primary culture (explants) for shoot multiplication and proliferation.

4.1.1. Induction of shoots from explants

The effect of BA and KIN on shoot proliferation and elongation in *Caladium bicolor* was investigated by adding different concentrations of BA and KIN to a basal MS medium (semi-solid). *In vitro* culture of shoot tip results 16.67% shoot induction at 1.0 mg/L BA whereas 13.33% shoot induction observed at 1.0 mg/L Kin. At control it was observed 3.33% shoot induction. Maximum number of shoot (5) initiated when MS media was supplemented with 1.0 mg/L BA and minimum (1) was at control. (Table 1).

Table 1. Effect of BA and Kin on shoot induction potentiality in *Caladium bicolor*

Name of phytohormone	Phytohormone concentration (mg/L)	Shoot induction potentiality		
		Explants cultured	Initiated shoot	Explants showing

		(Number)	(Number)	shoot induction (%)
Control	0	30	1	3.33
BA	0.25	30	2	6.66
BA	0.50	30	3	10.0
BA	0.75	30	2	6.67
BA	1.00	30	5	16.67
KIN	0.25	30	2	6.67
KIN	0.50	30	2	6.67
KIN	0.75	30	3	10.0
KIN	1.00	30	4	13.33

4.2. Sub-experiment 2. Combined effect of BA and IBA on shoot induction potentiality and shoot morphology in *Caladium bicolor*

4.2.1. Shoot induction potentiality

Table 2. Combined effect of BA and IBA on shoot induction potentiality in *Caladium bicolor*

Treatments	Treatment combination	Phytohormone concentration (mg/L)	Shoot induction potentiality			
			Explants cultured (Number)	Initiated shoot (Number)	Explants showing shoot induction (%)	Shoot induction (Days)
T_1	Control	0	30	1	3.33	85.50
T_2	BA+ IBA	0.25 +1.00	30	12	40.00	67.50
T_3	BA+ IBA	0.50+1.00	30	15	50.00	68.60
T_4	BA+ IBA	0.75+1.00	30	17	56.67	65.53
T_5	BA+ IBA	1.00+1.00	30	18	60.00	61.37
T_6	BA+ IBA	0.25+1.50	30	20	66.67	59.37
T_7	BA+ IBA	0.50+1.50	30	21	70.00	57.53
T_8	BA+ IBA	0.75+1.50	30	23	76.66	55.50
T_9	BA+ IBA	1.00+1.50	30	24	80.00	50.50
T_{10}	BA+ IBA	0.25+2.00	30	24	80.00	48.60
T_{11}	BA+ IBA	0.50+2.00	30	27	90.00	47.80
T_{12}	BA+ IBA	0.75+2.00	30	25	83.33	45.47
T_{13}	BA+ IBA	1.00+2.00	30	25	83.33	40.47
T_{14}	BA+ IBA	0.25+2.50	30	23	76.66	47.50
T_{15}	BA+ IBA	0.50+2.50	30	22	73.33	47.60
T_{16}	BA+ IBA	0.75+2.50	30	21	70.00	50.50
T_{17}	BA+ IBA	1.00+2.50	30	21	70.00	50.47
$LSD_{0.05}$						1.563
CV %						1.69

To identify the combined effect of BA and IBA at different concentration on shoot induction potentiality and days for shoot induction in *Caladium bicolor* was observed (Table 2). In each case 30 vials were cultured. Maximum percentage (90%) of explants showed shoot induction at 0.50 mg/L BA + 2.00 mg/L IBA and minimum percentage (3.33) was recorded at control. Minimum shoot induction

days (40.47) were recorded at BA 1.00 mg/L +2.00 mg/L IBA. and maximum (85.50) was recorded at control (Plate 3).

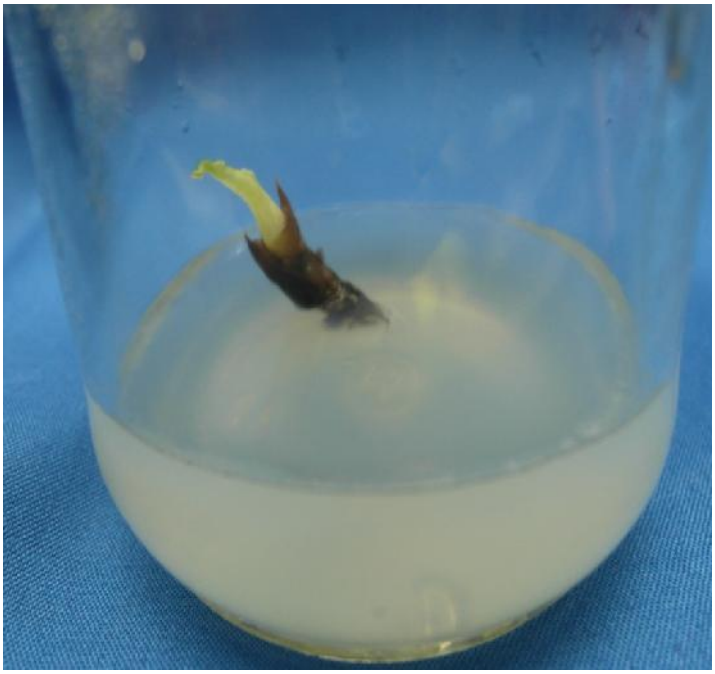

Plate 3. Shoot induced 40 days after inoculation when MS media was supplemented with 1.0 mg/L BA+2.00 mg/L IBA

4.2.2. Shoot morphology:

Table 3. Combined effect of BA and IBA on shoot morphology and shoot length in *Caladium bicolor*

Treatments	Name of the phytohormone	Phytohormone concentration (mg/L)	Shoot morphology			
			No. of shoots (3WAI)	No. of shoots (5WAI)	No. of shoots (8WAI)	Length of shoot (cm) 8 WAI
T_1	Control	0	1.367	2.400	3.240	2.200
T_2	BA+ IBA	0.25 +1.00	3.167	5.200	8.913	3.900
T_3	BA+ IBA	0.50+1.00	4.333	6.333	9.680	4.067
T_4	BA+ IBA	0.75+1.00	4.500	6.200	10.13	3.867
T_5	BA+ IBA	1.00+1.00	4.367	6.333	8.490	4.100
T_6	BA+ IBA	0.25+1.50	3.800	5.800	8.080	4.233
T_7	BA+ IBA	0.50+1.50	4.600	6.633	9.633	4.533
T_8	BA+ IBA	0.75+1.50	4.233	6.467	9.440	4.767
T_9	BA+ IBA	1.00+1.50	4.567	6.533	8.967	5.100
T_{10}	BA+ IBA	0.25+2.00	4.767	6.767	9.960	6.100
T_{11}	BA+ IBA	0.50+2.00	5.433	7.467	9.080	6.600
T_{12}	BA+ IBA	0.75+2.00	5.967	7.933	10.35	7.100
T_{13}	BA+ IBA	1.00+2.00	6.467	12.60	19.67	8.700
T_{14}	BA+ IBA	0.25+2.50	5.167	7.267	10.26	11.43
T_{15}	BA+ IBA	0.50+2.50	4.533	6.400	8.550	7.333
T_{16}	BA+ IBA	0.75+2.50	4.100	6.267	9.760	7.133
T_{17}	BA+ IBA	1.00+2.50	3.900	5.967	8.370	6.467
$LSD_{0.05}$			0.2624	0.3856	0.3481	0.1740
CV %			3.59	3.51	2.72	3.66

Combined effect of of BA and IBA showed significant variations on number of shoots at different time interval, length of shoot (cm) and number of leaves per shoot at 5% level of significance (Table 3). At 3 weeks after induction (WAI) maximum number (6.5) of shoots were recorded at 1.00 mg/L BA + 2.00 mg/L IBA and minimum (1.35) was recorded in control. Maximum (12.60) number of shoot (Plate 4) was observed when 1.00 mg/L BA was combined with 2.00 mg/L IBA and minimum (2.40) was recorded in control at 5 weeks after induction. Eight weeks after induction maximum number (19.67) of shoots were recorded (Plate 4) at 1.00 mg/L BA + 2.00 mg/L IBA and minimum

(3.24) was recorded in control. During the measurement of length of shoot maximum length (11.43 cm) and longest shoot (Plate 5) was observed at 0.25 mg/L BA + 2.5 mg/L IBA at 8 weeks after induction and minimum length (2.20 cm) was at control.

Plate 4. Maximum number of shoots produced from shoot tip cultured on MS medium supplemented with 1.0 mg/L BA and 2.0 mg/L IBA at 8 WAI

Plate 5. Longest shoots produced from shoot tip cultured on MS medium supplemented with 0.25 mg/L BA with 2.5 mg/L IBA at 8 WAI

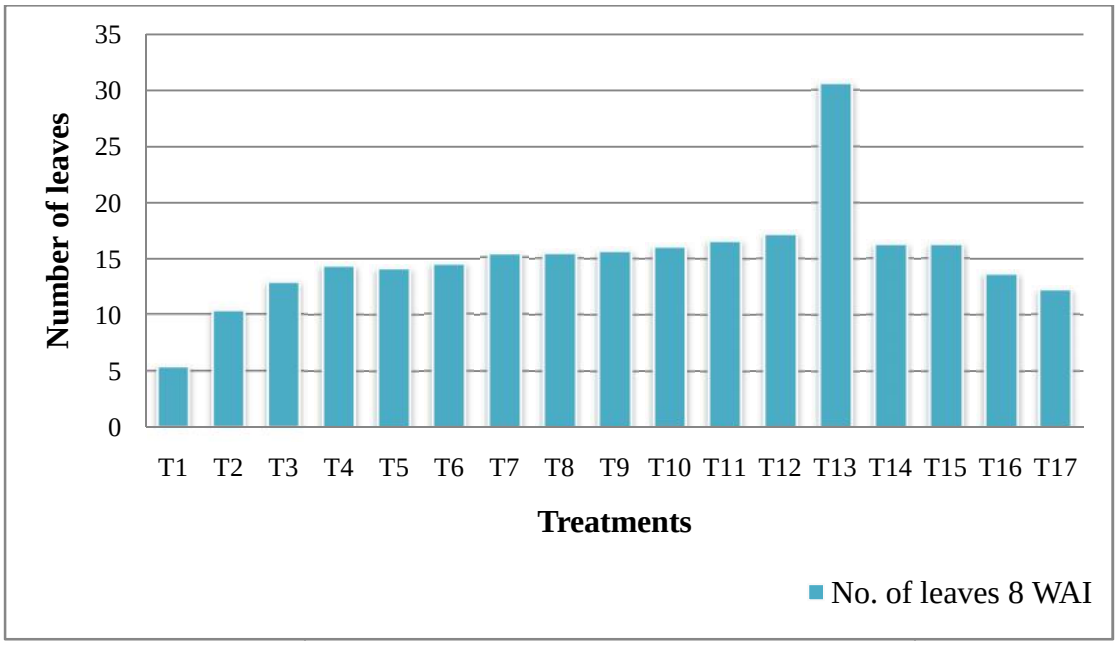

Figure 1. Combined effect of BA with IBA on leaf number in *Caladium bicolor*

Number of leaves were counted 8 weeks after induction, maximum (30.67) was recorded (Figure 1. and Plate 6.) at 1.00 mg/L BA + 2.00 mg/L IBA and minimum (5.43) was in control.

Plate 6. Maximum number of leaves produced from shoot tip cultured on MS medium supplemented with 1.0 mg/L BA with 2.0 mg/L IBA at 8 WAI

4.3. Sub-experiment 3. Effect of different concentrations of NAA, IAA and IBA on root formation potentiality and root morphology of the micropropagated shoots in *Caladium bicolor*

Table 4. Effect of rooting hormone NAA on root number and length in *Caladium bicolor* micropopagation

Treatments	Name of the rooting hormone	rooting hormone concentration (mg/L)	Root formation potentiality				Length of roots (cm)
			Explants showing root induction (%)	No. of roots 3 WAI	Noof roots 5 WAI	No. of roots 8 WAI	
T_1	Control	0	13.33	0.700	2.367	2.867	2.133
T_2	NAA	1.00	70.00	5.533	14.87	23.53	3.800
T_3	NAA	1.50	66.67	4.833	11.00	17.37	5.400
T_4	NAA	2.00	60.00	4.200	9.000	14.47	6.200
T_5	NAA	2.50	50.00	3.667	6.367	11.23	7.533
CV%				9.30	10.82	7.85	8.63
$LSD_{0.05}$				0.6406	1.716	1.984	0.7867

To investigate the efficacy of rooting hormone NAA different concentration was used (Table 4). Maximum (70.00 %) percentage of explants showed shoot induction at 1.00 mg/L concentration and minimum (13.33) was at control. Three weeks after induction maximum (5.53) roots were counted when NAA was used at 1.00 mg/L concentration and minimum (0.70) was at control. Five weeks after induction maximum (14.87) roots were counted at 1.00 mg/L concentration and minimum (2.37) was in control. Maximum (23.53) roots were observed at 1.00 mg/L concentration at 8WAI and minimum (2.87) was in control. Eight weeks after induction maximum average length (7.53 cm) of root was observed when

NAA was used at 2.50 mg/L concentration and minimum (2.13 cm) was in control.

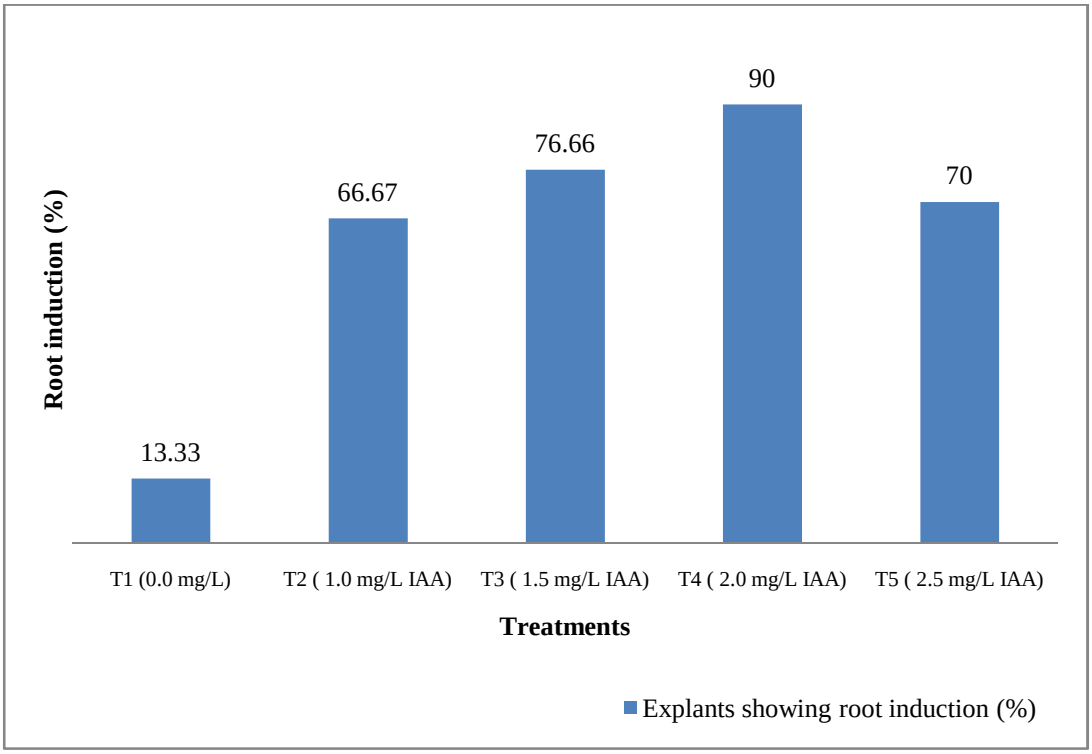

Figure 2. Effect of IAA on root induction percentage in *Caladium bicolor* micropopagation

To study the root induction percentage in *Caladium bicolor* different concentrations of IAA were used (Figure 2). Maximum (90.00%) root induction observed at 2.0 mg/L IAA followed by 76.66% at 1.5 mg/L IAA. Minimum (13.33%) percentage of root induction was observed at control.

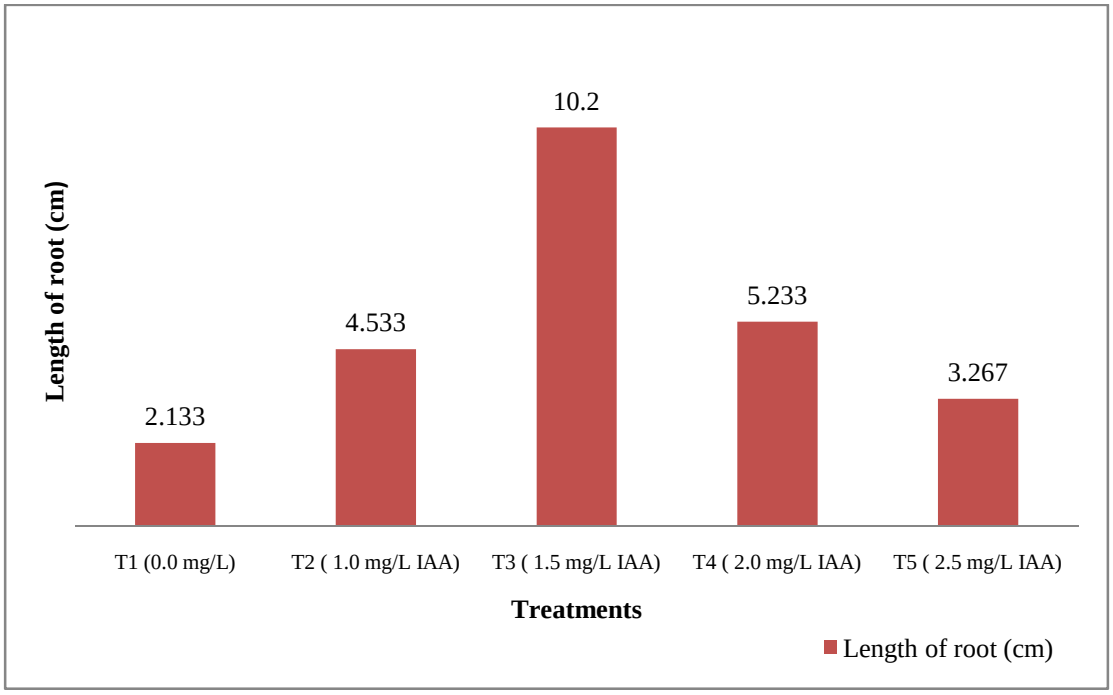

Figure 3. Effect of IAA on length of root in *Caladium bicolor* micropopagation

To identify the effect of IAA in root length in *Caladium bicolor* different concentration were practiced (Figure 3). Maximum (10.20 cm) average root length (Plate 7) was recorded at 1.50 mg/L IAA whereas minimum (2.13 cm) was at

control. Longest length (Plate 8) of root was observed also at 1.50 mg/L IAA 8 WAI.

Plate 7. Maximum average length of root observed from micropropagated shoot cultured on MS medium supplemented with 1.5 mg/L IAA at 8 WAI

Plate 8. Longest length root observed from micropropagated shoot culture when MS medium supplemented with 1.5 mg/L IAA at 8 WAI

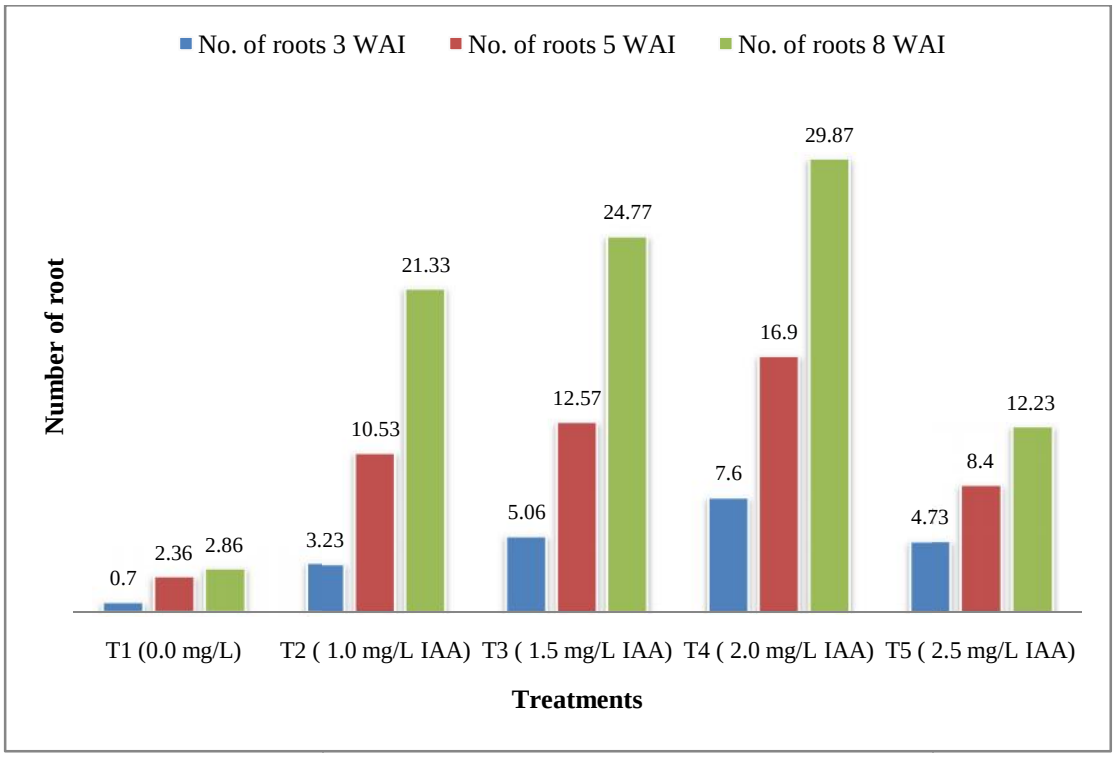

Figure 4. Effect of IAA on root number in *Caladium bicolor* micropopagation

To investigate the efficacy of IAA on root number in *Caladium bicolor* different concentration were used (Figure 4). Three weeks after induction maximum (7.60) roots were counted when IAA was used at 2.00 mg/L concentration and minimum (0.70) was at control. Five weeks after induction maximum (16.90) roots were counted at 2.00 mg/L concentration and minimum (2.37) was in control. Maximum (29.87) average roots were observed (Plate 9) at 2.00 mg/L concentration at 8 WAI and minimum (2.86) was in control.

Plate 9. Maximum average number of roots produced from micropropagated shoot cultured on MS medium supplemented with 2.0 mg/L IAA at 8 WAI

Table 5. Effect of IBA on root number and length in *Caladium bicolor* Micropopagation

Treatments	Name of the rooting hormone	rooting hormone concentration (mg/L)	Root formation potentiality				Length of roots
			Explants showing root induction (%)	No. of roots 3 WAI	No. of roots 5 WAI	No. of roots 8 WAI	
T_1	Control	0	13.33	0.700	2.367	2.867	2.133
T_2	IBA	1.00	50.00	3.200	7.400	12.80	3.433
T_3	IBA	1.50	66.67	3.867	8.467	15.67	5.233
T_4	IBA	2.00	70.00	4.033	11.33	19.37	6.533
T_5	IBA	2.50	83.33	5.533	13.57	22.87	4.367
CV%				11.17	10.98	6.23	11.46
$LSD_{0.05}$				0.7046	1.723	1.668	0.9042

Effect of IBA in root formation potentiality and root length was observed at different concentration of IBA (Table 5). Maximum (83.33 %) percentage of explants showed root induction at T_5 (2.50 mg/L) concentration and minimum (13.33) was at T_1 (control). Three weeks after induction maximum (5.53) roots were counted when IBA was used at 2.50 mg/L concentration and minimum (0.70) was at control. Five weeks after induction maximum (13.57) roots were counted at T_5 (2.50 mg/L) concentration and minimum (2.37) was in control. Maximum (22.87) roots were observed at 2.50 mg/L concentration at 8 WAI and minimum (2.867) was in control. Eight weeks after induction maximum average length (6.53 cm) of root was observed when IBA was used at 2.0 mg/L concentration and minimum (2.133 cm) was in control.

4.4. Sub-experiment 4. *Ex vitro* acclimatization and establishment of plantlets on soil

Table 6. Survial rate of *in vitro* regenerated plantlets of *Caladium bicolor*

Acclimatization	No. of plants transplanted	No. of plants survived	Percentage of survival rate
In growth chamber	30	30	100
Shade house with less humidity and indirect sunlight	30	26	87
In open atmosphere	26	24	92

Eight week after induction good number of root and shoot were found and plants were acclimatized in growth chamber, shade house and in open atmosphere. 30 plants were transplanted in growth chamber (Plate 10) where all plants were survived. Thus survival rate of plant in growth chamber was 100 %. In shade house 30 plants were transplanted , 26 survived and survival rate was 87 %. In open atmosphere 26 plants were transplanted 24survived (Plate 11) and survival rate was 92 %. So considering the survival rate it can be said that acclimatization potentiality of *Caladium bicolor* is satisfactory.

Plate 10. Sixty days old plantlet of *Caladium bicolor* transferred to growth chamber

Plate 11. Established plantlet of *Caladium bicolor* in open field

CHAPTER V

SUMMARY AND CONCLUSION

The experiment entitled "*In vitro* regeneration of *Caladium bicolor"* was conducted at the Biotechnology laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka during the period from October 2013 to July 2014. Three different sets of experiment were conducted and protocol were developed for *in vitro* regeneration. The first experiment was conducted to investigate the shoot induction potentiality of *Caladium bicolor* with single hormonal concentration. The second experiment was conducted to investigate the shoot induction potentiality and shoot morphology of *Caladium bicolor* with combined hormonal concentration. The third experiment was conducted to study the root formation potentiality and root morphology of *Caladium bicolor* with different hormonal concentration. The experiment was designed following Completely Randomized Design (CRD) with three replications.

In first experiment, individual effect of BA and KIN on shoot proliferation and elongation in *Caladium bicolor* was investigated. Maximum number of shoot (5) initiated when MS media was supplemented with 1.0 mg/L BA and minimum (1) was at control.

Maximum percentage (90.00%) of explants showed shoot induction when 0.50 mg/L BA was combined with 2.00 mg/L IBA and minimum percentage (3.33%) was recorded at control. In case of shoot induction duration in *Caladium bicolor* minimum (40.47) days required when 1.0 mg/L BA was combined with 2.00 mg/L IBA and maximum was recorded (85.50 days) in control (0.00 mg/L). Eight weeks

after induction maximum number (19.67) of shoots were recorded when 1.00 mg/L BA was combined with 2.00 mg/L IBA and minimum (3.24) was recorded in control. Number of leaves were counted 8 weeks after induction, maximum (30.67) was recorded when 1.00 mg/L BA was combined with 2.00 mg/L IBA and minimum (5.43) was in control.

Maximum (70.00 %) percentage of explants showed shoot induction at 1.00 mg/L NAA concentration. Maximum (90.00%) percentage of explants showed shoot induction at 2.00 mg/L concentration when IAA was used as rooting hormone. In case of IBA maximum (83.33 %) percentage of explants showed root induction at 2.50 mg/L concentration. In each case minimum (13.33%) percentage of explants showed root induction in control.

Three weeks after induction maximum 5.53 roots were counted when NAA was used at 1.00 mg/L concentration, 7.60 roots were recorded in case of IAA at 2.00 mg/L concentration and 5.53 roots were observed when IBA was used at 2.50 mg/L concentration. In every case minimum (0.70) number of roots was observed 3 WAI in control.

Eight weeks after induction maximum 23.53 roots were counted when NAA was used at 1.00 mg/L concentration, 29.87 roots were recorded in case of IAA at 2.00 mg/L concentration and 22.87 roots were observed when IBA was used at 2.50 mg/L concentration. Among the three rooting hormones IAA showed the best performance in case of shoot number and shoot length.

For acclimatization, plantlets were transplanted from culture media to soil in plastic pots in controlled environment, where the survival rate was 100%. After hardening, plantlets were transferred to shade house where the survival rate was 87%. Then the hardened plantlets were transferred to open field where the survival rate was 92%.

Recommendation

Following recommendations could be addressed based on the present experiment:

i. From the findings of the study it is very clear that the combination of 1.0 mg/L BA+2.0 mg/L IBA showed good performance in terms of shoot formation and 2.0 mg/L IAA showed good performance in root formation. In this experiment shoot induction took 40 days. However further intensive study needed to be carried out to evaluate different hormones at suitable doses to reduce the shoot induction time.

ii. For future research more doses of hormone combination can be taken as treatments with fewer intervals which will give us specific result.

iii. At the same time, along with BA, KIN, NAA, IAA and IBA other types of cytokinin and auxin be taken into trial.

iv. Except shoot tip culture, petiole, leaf and callus culture could be practised.

v. Precise and details investigation on influence of other factors such as different elicitors, antioxidants should be considered.

CHAPTER VI

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CHAPTER VII

APPENDICES

Appendix I. Composition of Duchefa Biochemie MS (Murashige and Skoog, 1962) medium including vitamins

Components	Concentrations (mg/L)	Concentrations
Micro Elements	**mg/L**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements	**mg/L**	**mM**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61
Vitamins	**mg/L**	**µM**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30

Total concentration of Micro and Macro elements including vitamins: 4405.19 mg/L

Manufacturing Company: Duchefa Biochemie, Netherland.

Appendix II. Analysis of variance on days for shoot induction with combination of BA and IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	6008.279	375.517	423.516	0.0000	1.69	1.563
Within	34	30.147	0.887				
Total	50	6038.426					

Appendix III. Analysis of variance on number of shoot 3 WAI with combination of BA and IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	60.162	3.760	148.655	0.0000	3.59	0.2624
Within	34	0.860	0.025				
Total	50	61.022					

Appendix IV. Analysis of variance on number of shoot 5 WAI with combination of BA and IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	180.333	11.271	209.022	0.0000	3.51	0.3856
Within	34	1.833	0.054				
Total	50	182.166					

Appendix V. Analysis of variance on number of shoot 8 WAI with combination of BA and IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	451.572	28.223	418.158	0.0000	2.72	0.4295
Within	34	2.295	0.067				
Total	50	453.866	0.011				

Appendix VI. Analysis of variance on length of shoot 8 WAI with combination of BA and IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$

Between	16	244.333	15.271	1416.018	0.0000	1.82	0.1740
Within	34	0.367					
Total	50	244.699					

Appendix VII. Analysis of variance on number of leaves 8 WAI with combination of BA and IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	1157.905	72.369	201.684	0.0000	3.96	0.9942
Within	34	12.200	0.359				
Total	50	1170.105					

Appendix VIII. Analysis of variance on number of roots 3 WAI with NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	41.577	10.394	83.825	0.0000	9.30	0.6406
Within	10	1.240	0.124				
Total	14	42.817					

Appendix IX. Analysis of variance on number of roots 5 WAI with NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	266.884	66.721	74.967	0.0000	10.82	1.716
Within	10	8.900	0.890				
Total	14	275.784					

Appendix X. Analysis of variance on number of roots 8 WAI with NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	701.956	175.489	147.552	0.0000	7.85	1.984
Within	10	11.893	1.189				
Total	14	713.849					

Appendix XI. Analysis of variance on length of roots 8 WAI with NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$

Between	4	53.024	13.256	70.762	0.0000	8.63	0.7867
Within	10	1.873	0.187				
Total	14	54.897					

Appendix XII. Analysis of variance on number of roots 3 WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	77.273	19.318	100.616	0.0000	10.27	0.7972
Within	10	1.920	0.192				
Total	14	79.193					

Appendix XIII. Analysis of variance on number of roots 5 WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	345.577	86.394	75.918	0.0000	10.51	1.716
Within	10	11.380	1.138				
Total	14	356.957					

Appendix XIV. Analysis of variance on number of roots 8 WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	1379.284	344.821	406.948	0.0000	5.05	1.984
Within	10	8.473	0.847				
Total	14	1387.757					

Appendix XV. Analysis of variance on length of roots 8 WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	115.523	28.881	120.003	0.0000	9.67	0.7867
Within	10	2.407	0.241				
Total	14	117.929					

Appendix XVI. Analysis of variance on number of roots 3 WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	37.433	9.358	62.389	0.0000	11.17	0.7046
Within	10	1.500	0.150				
Total	14	38.933					

Appendix XVII. Analysis of variance on number of roots 5 WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	217.343	54.336	60.597	0.0000	10.98	1.723
Within	10	8.967	0.897				
Total	14	226.309					

Appendix XVIII. Analysis of variance on number of roots 8 WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	699.131	174.783	207.910	0.0000	6.23	1.668
Within	10	8.407	0.841				
Total	14	707.537					

Appendix XIX. Analysis of variance on length of roots 8 WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	33.903	8.476	34.268	0.0000	11.46	0.9042
Within	10	2.473	0.247				
Total	14	36.376					