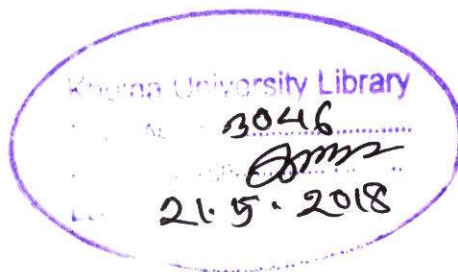


**Optimization of *in vitro* propagation and tuberization
of potato (*Solanum tuberosum* L.) using apical
meristem.**



A Thesis Submitted

By

Shukla Saha

Examination Roll No. MS-090708

Session: 2008-2009

Submitted to

**Biotechnology and Genetic Engineering Discipline, life science school,
Khulna University, Khulna in partial fulfillment of
the requirements for the degree of 18 months
'Master of Science (MS) in Biotechnology and Genetic Engineering'**

**Biotechnology and Genetic Engineering Discipline,
Life Science School, Khulna University,
Khulna-9208, Bangladesh.**

Optimization of *in vitro* Propagation and Tuberization of Potato (*Solanum tuberosum* L.) Using Apical Meristem.

**A Thesis submitted
By**

Shukla saha

Examination Roll No. MS-090708

Session: 2008-2009

Approved as to the style and content by

Mahmed
20.07.2011

Professor Dr. Mahtalat Ahmed

Supervisor
Agrotechnology Discipline,
Khulna University,
Khulna-9208, Bangladesh.

Raihan 20.7.11

Professor Md. Raihan Ali

Co-Supervisor
Biotechnology and Genetic
Engineering Discipline,
Khulna University,
Khulna-9208, Bangladesh.

Khondoker 20/07/11

Prof. Dr. Khondoker Moazzem Hossain

Chairman,
Examination Committee, and
Head
Biotechnology and Genetic Engineering Discipline,
Life Science School, Khulna University,
Khulna-9208, Bangladesh.

DECLARATION

It is stated that, this research work is a new one and this thesis has not been previously or concurrently submitted for other degree at Khulna University or other institutions. No literature was copied directly from other scientific papers except for quotations and citations which have been fully acknowledged. Nobody is permitted to publish this thesis in any peer reviewed journal except the author and the supervisor.

Shukla Saha
20.07.2011
Shukla Saha

Roll Number: MS-090708

Session: 2008-2009



*Dedicated to
My Parents*

CONTENTS

TOPIC	PAGE NO.
Acknowledgement	I
Abstract	II - III
List of Tables	IV
List of Figures	VI
Chapter One: Introduction	1-4
Chapter Two: Literature Review	5-12
Chapter Three: Materials and Methods	13-23
3.1. Materials	13
3.2. Methods	14
3.2.1. Preparation of stock solutions for MS medium	14
3.2.1.1. Macro nutrient stock	14
3.2.1.2. Micro nutrient stock	14
3.2.1.3. Iron stock	14
3.2.1.4. Vitamin stocks	15
3.2.2. Growth hormone [Gibberellic acid (GA ₃), Napthalene acetic acid (NAA) and Benzyl amino purine (BAP)] stocks	15
3.2.3. Preparation of culture media	15
3.2.4. Growth regulators used for meristem proliferation and multiplication of plantlets	16
3.2.5. Constituents of media for microtuberization of potato	17
3.2.6. Substrates used in pots for minituber formation	18
3.2.7. Sterilization	18
3.2.7.1. Sterilization of glass wares and instruments	18
3.2.7.2. Sterilization of culture room and transfer area	18
3.2.8. Experimental design	19
3.2.9. Procedure for meristem culture establishment	19
3.2.9.2. Inoculation of explants	20

3.2.9.3. Incubation of the cultures	20
3.2. 10. Procedure for multiplication of regenerated plants	20
3.2.11. Procedure for multiplication of regenerated plants obtained from shoot buds of different positions in plant body	21
3.2. 12. Procedure for microtuberization	21
3.2. 13. Procedure for minituber production	21
3.2.14. Parameters studied and data collection	22
3.2.15. Analysis of data	23
Chapter Four: Results and Discussion	24-34
4.1. Results of the experiment	24
4.1.1. Meristems response in culture	24
4.1.2. Effect of different sucrose concentrations on <i>in vitro</i> microtuberization of potato	29
4.1.3. Effect of different substrates on <i>Ex vitro</i> performance of plants and minituber production of potato	30
4.2. Discussion	32
Chapter Five: Summary and Conclusion	35-37
References	38-43
Appendices	44-49
Figures	50-55

ACKNOWLEDGEMENT

At first, the author would like to express her greatest and deepest gratitude to the supreme of the universe, almighty God to whom all praises go for enabling the author to complete this thesis with sound condition.

The author would like to express deepest sense of gratitude, sincere appreciation and indebtedness to her respected supervisor Professor Dr. Mahtalat Ahmed, Agrotechnology Discipline, Life Science School, Khulna University, Khulna, for her materialistic support, keen interest, scholastic guidance, valuable critique, active cooperation, adoring supervision, generous help, demonstrative feeling, uninterrupted encouragement from the very beginning to the end of research work and thesis writing.

The author extend her profound gratitude and heartiest appreciation to her co-supervisor Professor Md. Raihan Ali, Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna, for his cordial inspection, scholastic counseling, productive suggestions and all sorts of helps during the research work.

No words can express my deep sense of reverence for Professor Dr. Monirul Islam, Agrotechnology Discipline, Khulna University, Khulna, who with his able guidance, endless patience, altruistic help, constructive criticism and affectionate attitude, made me work on the problem. I am highly obliged to him for his cordial encouragement throughout the period of investigation.

The authors wish to express earnest gratitude and thanks to all honorable teachers of Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna, for their blessing and direct or indirect support during the research work.

Appreciation is also extended to Md. Samsul Alam, Section Officer (lab.), Agrotechnology discipline who cooperate with the researchers in providing necessary information, timely assistance, mental support and helping during the research work.

The authors are indebted to her lab-mates, friends, parents and relatives for their encouragement, inspiration, constructive criticism, assistance and helpful contribution during the research work.

July, 2011

The Author

ABSTRACT

Highest frequency of shoot induction (3.33%) from meristems was occurred in the Smart variety on the media containing $5 \text{ mg l}^{-1} \text{ GA}_3 + 0.5 \text{ mg l}^{-1} \text{ BAP} + 0.5 \text{ mg l}^{-1} \text{ NAA}$ and $6 \text{ mg l}^{-1} \text{ GA}_3 + 0.5 \text{ mg l}^{-1} \text{ BAP} + 0.5 \text{ mg l}^{-1} \text{ NAA}$ whereas, no. of branch per plant and average shoot length were higher at $6 \text{ mg l}^{-1} \text{ GA}_3$. the Smart variety responded well in rooting (20%) and 4% of the inoculated meristems of this variety produced shoots with an average shoot length of 1.48. Other two varieties viz. Natura and Laura did not respond at all to shoot formation from inoculated meristems. Maximum rooting (80%) was noticed in the variety Smart on the medium containing GA_3 - 2.0, BAP- 1.0 and NAA- 0.5 mg l^{-1} . Plantlets formed *in vitro* from the variety Smart were multiplied clonally by shoot buds. $5 \text{ mg l}^{-1} \text{ GA}_3 + 0.1 \text{ mg l}^{-1} \text{ NAA}$ was found better in highest shoot length (91.45 mm) and root length (35.28 mm). Shoot buds of different positions in plant body were also investigated for *in vitro* clonal multiplication. In all respects, apical buds show better performance. The highest shoot length was 64.26 mm observed in apical bud whereas, the lowest in axillary bud - 4. Highest root length and healthy plants were also found in apical bud. Percentage of survivability of microtuber producing plants also increased with the increase of sucrose concentration. Best survival rate and average diameter of tuber per plant was found at 10% sucrose. Minituber production on direct field showed best performance. It was observed that after 30 days, maximum shoot length per plant (188.87 mm) was found on the direct field whereas the lowest shoot length per plant (69.32 mm) was on sand. Lowest weight of tuber per plant was recorded on the substrates containing coconut dust. The highest weight of tuber was found in direct field. In case of, Average diameter of tuber per plant, 25% soil+ 75% coconut dust showed minimum result and on the other hand, in direct field, large size of tubers were found.

LIST OF TABLES

Table No.	Title of Table	Page No.
1	Combinations and concentrations of growth regulators used for meristem proliferation treatments.	16
2	Different levels of GA ₃ in combination with NAA (0.1 mg l ⁻¹) used for multiplication of regenerated plants.	16
3	Combined effect of plant growth regulators on survivability of explants and some growth parameters of regenerated plants in potato shoot tip culture	25
4	Effect of potato varieties on meristem proliferation and further development	26
5	Effect of various combinations and concentrations of growth regulators on growth parameters of meristems of three potato varieties	27
6	Effect of different concentrations of GA ₃ in combination with NAA on multiplication of regenerated plants of variety Smart.	28
7	Performance of plantlets obtained from apical and axillary buds	29
8	Effect of different sucrose concentration in presence of BAP (5 mg l ⁻¹) and CCC (500 mg l ⁻¹) on <i>in vitro</i> microtuberization from cultured shoot tips.	30
9	<i>Ex vitro</i> minituber production using different substrates.	31

LIST OF FIGURES

Figure No.	Title of Figure	Page No.
1.	Plant indicating apical and axillary buds.	17
2.	Potato sprouts.	19
3.	Excision of meristems under a binocular dissecting microscope.	19
4.	Meristem isolation from potato sprouts.	57
5.	Shoot and root formation from isolated meristem at 6 mg l ⁻¹ GA ₃ + 0.5 mg l ⁻¹ BAP + 0.5 mg l ⁻¹ NAA.	57
6.	Plantlets formed <i>in vitro</i> from the variety Smart at 5 mg l ⁻¹ GA ₃ + 0.1 mg l ⁻¹ NAA	57
7.	Plantlets formed <i>in vitro</i> from apical buds.	57
8.	Microtuber formation at 8% sucrose + 5.0 mg l ⁻¹ BAP + 500 mg l ⁻¹ CCC.	58
9.	Microtuber formation at 7% sucrose + 5.0 mg l ⁻¹ BAP + 500 mg l ⁻¹ CCC.	58
10.	Microtuber formation at 10% sucrose + 5.0 mg l ⁻¹ BAP + 500 mg l ⁻¹ CCC.	58
11.	Harvested Microtubers from <i>in vitro</i> grown plantlets.	58
12.	Transferred plants into plastic pots from <i>in vitro</i> grown plantlets were kept into glass jar for 24 hours.	59
13.	Transferred plants into green house after 24 hours.	59
14.	Plants grown on field directly.	59
15.	Harvested minitubers.	59
16.	Minituber formation from different substrates in <i>ex vitro</i> condition.	60
17.	Potato producing areas in the Bangladesh.	61
18.	Potato producing areas in the world.	62

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

Potato (*Solanum tuberosum* L.), belongs to the Solanaceae family, which encompasses about 90 genera and 2,800 species. The genus *Solanum*, has about 2,000 species and includes all tuber-bearing wild species as well as *Solanum tuberosum*. Potato is one of the most economically important annual crops. It is the most widely distributed crop in tropical and subtropical zones of the world. (<http://www.potato2008.org/en/aboutiyp/index.html>).

Potato is a short day, cool season and C₃ plant with a low light saturation point. It is known as herbaceous, succulent, dicotyledonous plant with alternate stolon underground and alternate leaves on the stem above ground; stems are about 30-100 cm tall. A potato plant can have three kinds of stems including sprouts (leafy stems), stolons and tubers. Tubers are underground, fleshy stems with eyes and they are suitable for ware, food processing, seed, animal feed and non-industrial use. (Otroshy, 2006.)

The origin of cultivated potatoes lies in the high Andes of South America and the costal strip of central to southern Chile (Hawkes, 1990). After the first potatoes were brought to Europe in the late 16th century, this crop plant was distributed and utilized all over the world mostly in temperate regions (Hawkes, 1990). Today over 4000 varieties of *Solanum tuberosum* ssp. *tuberosum* exist (Hils and Pieterse, 2005). Because of the high number of varieties and related species, potato is known to have the richest genetic diversity of any cultivated plant (FAO, 2007). Conservation of genetic resources in gene banks is important, because of their high value as breeding material, for research, industry and private needs.

Potato is one of the most frequently cultivated species on earth and ranks on place four in produced crops after maize, rice and wheat (FAOSTAT, 2007; Spooner and Bamberg, 1994). Among cereals and the root and tuber crops potato has the highest yield with 16.7 kg ha⁻¹ (FAO, 2007). The cultivated potato is used for food, feed, medicine, processed foods (potato chips, alcoholic beverages) and industrial products

like plastic polymers (from potato starch). Thus, potato is clearly a crop of considerable world importance.

Potato contains very low fat, about 5 percent of the fat content of wheat, and one-fourth of the calories of bread, while boiled potato has more protein than maize, and nearly twice the amount of calcium. A single potato tuber (of medium size) has about half the daily adult requirement of vitamin C whereas some other staples like rice and wheat have none (CIP website at <http://www.cipotato.org>). The potato is a wholesome food with all the extremely important and necessary dietary constituents, which are needed for health and growth. Compared to other roots and tubers and also many cereals, potato tubers have a high ratio protein to carbohydrates with a high nutritional value of the protein (Sarkar *et al.*, 1998).

In some countries true potato seed (TPS) is used as planting material in a seed potato production scheme. Size of each true seed is 1.2 – 1.8 mm and the weight of 100 seeds is only 50 – 100 mg (Struik and Wiersema, 1999). In China, botanical seeds have been used for many years (Akita *et al.*, 1994). TPS can solve some of the problems associated with conventional systems. True potato seed has advantages such as: ease to transport and store (it can be stored at room temperature and dark conditions for many years), lower cost, less transmission of diseases and pests (Hils *et al.*, 2005 and Khurana *et al.*, 2003). When TPS is used a large amount of potato tubers which would have been used for potato production and multiplication can now be used for other purposes, such as food (Yoon *et al.*, 2004 and Gopal, 1980). However, TPS also has some disadvantages, including high labour requirement, less uniformity in plant type and maturity for crops grown from TPS, and more variation in colour and shape and dry matter content of tubers produced from TPS. Moreover, potato seedlings are weak and can be damaged easily by environmental stress and pests (Khurana *et al.*, 2003).

Recently some new multiplication techniques have been developed and they are now used all over the world. One of these new techniques is rapid multiplication (or micropropagation or tissue culture technique). This technique is now widely used in many different countries. Rapid multiplication is very flexible and gives a high rate of multiplication. It also provides seed potato tubers free from seed borne diseases

(Vandenbussche *et al.*, 1999). Rapid multiplication can solve some of the problems associated with the conventional multiplication system (Struik & Wiersema, 1999). Tissue culture revolutionized seed potato production in some countries such as Vietnam and doubled potato yield and potato area (Ward, 1993). A large number of virus-free potato microplants can be obtained by the micropropagation technique (Khurana *et al.*, 2003).

Higher rate of multiplication, disease-free material (including plantlets, transplants, microtubers and minitubers), more convenient storage and transport, and a small demand for space during multiplication are known as advantages of rapid multiplication. Moreover, multiplication can be done whole year round (Struik & Wiersema, 1999). *In vitro* propagation methods using meristem tips, nodal cuttings and micro tubers are more reliable for maintaining genetic integrity of the multiplied clones since de-differentiation and the subsequent organogenesis/ embryogenesis with the accompanying genetic changes have been reported (Wang and Hu, 1982). Meristem culture provides a reproducible and economically viable method for producing pathogen free plants. As meristem tips are free from viruses, elimination and generation of virus free plants are possible through meristem culture (Hils, 2005).

In Bangladesh, potato is an important vegetable and supplementary food crop. Tubers, the underground modified stems are generally used for the propagation of potato. In case of Bangladesh the total area of cultivation, approximately 0.31 million hectares, with a total production of 4.3 million tons. In spite of high demand, Bangladesh is not in a condition to produce potato at world level, still in Bangladesh average potato yield ha^{-1} (13.9 tons) is lower than average world production ha^{-1} (15.7 tons) (<http://www.potato2008.org/en/world/asia.html>). In Bangladesh low yield of potato is due to the use of degenerated low quality seeds. The presence of viral diseases is an important reason attributed to low yield of potato varieties.

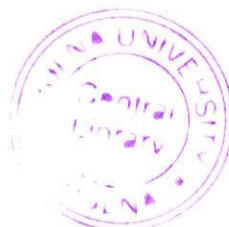
The major reasons behind this are lack of suitable genotypes that can resist some specific diseases (Late blight, Early blight, Black leg, Powdery mildew, Powdery scab, Leaf roll virus, and Purple top) and insect (Colorado potato beetle, Potato tuber moth, Green peach aphid, Beetle leafhoppers, Trips, and Mites), use of local

unimproved varieties, environmental stress, poor crop management and proper research and also lack of supply of certified seed potatoes. (Pruski, 2007)

Among all the biotic agents' viruses are the most dangerous for potato production. A single plant of potato may be infected with four to five viruses. Viral infected plants characterized by a decrease in vigor, productivity and susceptible to other pathogens. Some common dangerous potato viruses are Potato virus Y (PVY), Potato virus X (PVX), Potato virus S (PVS), Potato leaf roll virus (PLRV), Potato mosaic virus (PMV), Potato leaf curl virus (PLCR). The occurrence of potato viruses, including Potato virus Y (PVY), was observed in commercial fields developing in a single and mixed infections (<http://scialert.net/abstract/?doi=ijv.2009.64.76>). In all potato growing regions the availability of high quality clean seed tuber has been the most limiting owing to the conventional clonal propagation that favors disease build-up that drastically reduces yield.

The present research work has been undertaken to fulfill the following specific objectives:

1. To study the effect of different combinations and concentrations of BAP, NAA and GA₃ for massive proliferation and subsequent morphogenesis of potato meristems.
2. To study the effect of varietal differences on proliferation and morphogenesis of potato meristem *in vitro*.
3. To select appropriate concentration of GA₃ in presence of NAA for *in vitro* clonal propagation of regenerated plants.
4. To evaluate the performance of regenerated plantlets obtained from shoot buds of different positions in plant body.
5. To choose the most effective sugar concentration in *in vitro* microtuberization from cultured shoot tips and
6. To select the best substrates for minituber production from *ex vitro* transferred plantlets.



CHAPTER TWO

REVIEW OF LITERATURE

CHAPTER TWO

REVIEW OF LITERATURE

Tissue culture is a method based on vegetative propagation of planting materials (Shibli *et al.*, 2004). It involves *in vitro* multiplication of plant material under aseptic and controlled environmental conditions. Elementary methods of tissue culture were developed in the 1950's (Halmagyi, 2005). It assists the storage of disease free collections and facilitates easier maintenance and distribution of germplasm.

Tissue culture techniques are economically important for propagating elite plants, genetic upgradation and transformation. Plant tissue culture offers new ways for the improvement of crop plants (Senula *et al.*, 2007). *In vitro* propagation of plants holds tremendous potential for the production of high quality plant (Midmore, 1992).

Tissue cultured propagules are produced under controlled environment and the nutrients in the growing media are far in excess of those in the soil (Dumet, 2000). Such an environment causes developmental distortions and may repress or modulate several metabolic pathways. In addition, *in vitro* plantlets have small juvenile leaves with reduced photosynthetic capacity and malfunctioning stomata (Pruski, 2003). Hence, these plantlets need to be hardened gradually in order to improve survival upon transfer to soil (Leifert, 1992).

Tissue culture techniques open up many possibilities for sustainable food security and improvement of crops (Yoon *et al.*, 2004). The systems are capable of creating genetic variability and producing plants with novel characters (Roca, 1978), which could be more favorable than the existing crop varieties. Apart from that, meristem culture techniques, which were first developed by Robbins in 1972 (Struik *et al.*, 1999).

Micro propagation is the alternative to conventional propagation of potatoes (Chandra *et al.*, 1994). *In vitro* propagation methods using meristem tips, nodal cuttings and microtubers are more reliable for maintaining genetic integrity of the multiplied

clones since de-differentiation and the subsequent organogenesis/ embryogenesis with the accompanying genetic changes have been reported (Wang and Hu, 1982). Meristem culture provides a reproducible and economically viable method for producing pathogen free plants. As meristem tips are free from viruses, elimination and generation of virus free plants are possible through meristem culture (Zhao and Lim, 2005).

Meristem culture was pioneered by Pennazio (1976) and usually involves the removal of the meristem and subsequent culture on a nutrient medium, generally shiny special dome-like structure measuring less than 0.1 mm in length and only one or two pairs of the youngest leaf primordia, most often excised from the shoot apex (Chawla, 2002).

Some authors recommended using apical meristem of 0.25-0.3 mm length (Tailor *et al.*, 2004) although Wang and Hu (1982) proposed the size of the isolated meristem of 0.3 to 0.7 mm in length for successful elimination of virus from potatoes. Meristem culture has been used successfully in the removal of viruses from many plants e.g. Potato, Sugarcane, Strawberry, etc. (Aburkhes, *et al.*, 1984). This pioneering work on meristem culture has been extensively used for production of pathogen free potato plants in various countries (Abbott, 1986; Anonymous, 1992 and Bajaj, 1981).

Potato is usually propagated asexually by means of tubers, that is, the underground stems of the plant. However, with the conventional method of vegetative propagation, potatoes are often prone to pathogens such as fungi, bacteria and viruses, thereby resulting in poor quality and yields. Consequently, much attention has been focused on the *in vitro* production of virus-free potatoes (Danielle *et al.*, 2003).

Due to the fact that it takes many months for meristems to become plantlets, some researchers have tested the application of chemical products (chemotherapy) that reduce or inhibit viral multiplication. In the case of potato, three of the most important viruses, PVX, PVS, and PVY, have been eradicated by adding Ribavirin to the culture media and isolating the axillary buds (Griffiths *et al.*, 1990).

Many potato plants contain systemic viruses which substantially reduce their potential yield and quality. It is therefore, important to produce virus free stocks which can be multiplied. Generally, highly meristematic tissue of a virus infected plant remains free from virus due to their rapid mitotic activity. The vascular bundle is absent in meristematic region, and without vascular bundle virus cannot move and it contains auxin (IAA) with high concentration. Lizarrage, *et al.*, 1986 used ribavirin treatment for eradicating potato virus X (PVX) by shoot tip culture. Frickey *et al.*, 2004 reported that inclusion of ribavirin in tissue culture media resulted in a high frequency of elimination of the potato virus complex X, Y, S, and M. Mahmoud *et al.*, 2009 cultured axillary bud for eradicating potato virus Y (PVY). For potato Yu *et al.*, 2000 reported PVX, PVY, and PLRV elimination in approximately 40% of regenerated plants.

Chemotherapy is being used in potato as an alternative to thermotherapy. An analog to the nucleoside, virazole, has shown variable results when applied to potato plants by aspersion or in hydroponic culture, followed by a meristem culture. Addition of 100 mg l⁻¹ of virazole to the meristem culture media has been successful for the eradication of PVS, PVX, and PVY, but not of PLRV (Griffiths, 1990).

Antibiotics are only used in vegetative tissue cultures when the microorganisms can not be eliminated using other methods. These are expensive, and none of them are effective enough to control all the contaminant organisms. Before using antibiotics, elimination of all possible sources of contamination is recommended (Reed and Tanprasert 1995).

Callus tissue means an unorganized proliferative mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificially prepared nutrient medium in glass vials under controlled experimental conditions (Danielle, 2003). Plant may be regenerated directly or indirectly through callus formation. By changing the amounts and types of hormones in the culture medium, the cells can be stimulated to develop into shoots and roots. In potato propagation callus culture has been used for creation of genetic variants and screening of cells *in vitro* for types with useful characteristics (Seong, *et al.* 2007).

Shoot tip culture may be described as the culture of terminal (0.1 - 1.0 mm) portion of a shoot comprising the meristem (0.05-0.1 mm) together with primordial and developing leaves and adjacent stem tissue (Dumet, 2000).

Shoot tip cultures are routinely used for the propagation of many plant species and the storage of such cultures may be an alternative method for the maintenance of material in gene-banks (Halmagyi, 2005). The advantages include a considerable degree of protection from environmental hazards and the possibility of elimination of viral infections. Storage of over decades together with the maintenance of genetic stability could be achieved by cryopreservation of the shoot tips in liquid nitrogen (-196 °C). In collaboration with the International Potato Centre, Peru (CIP) it has been shown that a wide range of Andean potato species can be propagated by shoot tip culture methods (http://aob.oxfordjournals.org/cgi/pdf_extract/42/5/1227).

Cultivated potatoes are maintained vegetatively, because they produce highly heterozygous seeds (Dumet *et al.*, 2000). For this reason collections are conserved clonally for preservation of individual genotypes. The simplest method of maintenance is cultivation in the field, where genetic integrity of the varieties can be verified directly (Spooner *et al.*, 1994).

Potato plantlets do not require exogenous hormone for rooting. In fact, they can be propagated, elongated and rooted on a single hormone-free medium. As soon as rooted plantlets are obtained, they are transferred to pots in greenhouses. Another approach consists of planting the rooted plantlets at very high densities to obtain minitubers and use the latter for subsequent field multiplication (Otroshy, 2006). The crop is usually propagated asexually by means of tubers, that is, the underground stems of the plant. However, with the conventional method of vegetative propagation, potatoes are often prone to pathogens such as fungi, bacteria and viruses, thereby resulting in poor quality and yields. Consequently, much attention has been focused on the *in vitro* production of virus-free potatoes (Roca *et al.*, 1978).

Phytohormones or their synthetic counterparts are required either singly or in combination to initiate and maintain cell division. The concentration and ratio of hormones may vary from plant to plant and should be standardized for a particular

plant tissue. The auxins that are commonly used in culture media are IAA (Indole-3-acetic acid), 2, 4-D (2, 4-dichloro phenoxyacetic acid), NAA (Naphthalene acetic acid), Indole butric acid (IBA) are widely used, of these NAA is considered more effective due to its better stability. In order to achieve rapid multiplication, addition of GA₃ to the culture media has to be improving growth and development of shoots (Roset and Bokelmann, 1976).

Cytokinin are involved both local and long distance signaling. These are transported within the xylem. Cytokinin act in concert with auxin that stimulates cell expansion. BAP is a first-generation synthetic cytokinin which elicits plant growth and development responses. (Chawla, 2002)

The temperature is usually kept constant at 24-26°C for shoot tip culture. Sometimes, depending on the experimental species a lower (18°C) or higher temperature (30°C) is chosen. Badoni and Chauhan (2009) incubated the culture media at 25±1°C and 16 h photoperiod. The optimal temperature for *in vitro* growth and development is generally 3-4°C higher than *in vivo*. Since the temperature within the test tube is 3-4°C higher than the growth room, due to the warming effect, the growth room temperature can be maintained at the optimal for *in vivo* plant growth. Behnke (1993) used 28°C and 14 h photoperiod for incubation. Gebre and Sathyanarayana (2001) incubated the culture at 26 ± 3°C and 16 h photoperiod.

Light is a very complicated factor, as well as day-length, irradiance and quality are of special importance for shoot tip culture. Little is known about the affect of day-length on *in vitro* cultures; the usual day-length chosen is 14-16 h, although continuous light is also used. Zaman *et al.* (2001) incubated the test tubes at 23-24°C in continuous light of 100 lux intensity and incase regeneration of plantlets they used 22-25°C at a light intensity of 2500 lux.

In some cases growth takes place in continuous darkness (e.g. callus, dark requiring orchid seeds). Behnke (1993) used white light of about 3000 lux, the callus initiation was obtained in dark. Growth usually takes place at an irradiance of 80-150 ($\mu\text{mol m}^{-2} \text{S}^{-1}$). Khan and Rabbani (1999) kept the cultures in a growth room on the shelves at 25±2°C illuminated with cool light from 1.83 m fluorescent tubes for 16 hours daily with a light intensity of 1500 lux.

In vitro tuber production is called microtuberization. Incubation of the nodal segments from regenerated shoots on MS media containing various concentrations of growth regulators and sucrose produce microtubers at 18°C in complete dark. There are many advantages to using potato microtubers rather than natural tubers. Potato microtubers are completely pathogen free in terms of seed borne diseases. Because of their small size and weight, microtubers have tremendous advantages in terms of storage, transportation and farm mechanization, thus dramatically decreasing the handling cost. Hoque *et al.* (2003) used shoot tip into agar solidified MS medium containing various hormone supplements. Ziv and Shemesh (2002) used single node stem segments in liquid medium in the presence of ancymidol.

For *in vitro* tuber production different protocols were used to induce microtubers, but generally the nutrient media with 80 g l⁻¹ sucrose, 6 g l⁻¹ agar were used, 500 ppm of Chlorocholine chloride (CCC) will be added to the medium after autoclaving (cytokinins and other substances can also be used) to induce microtuber production, pH was adjusted at 5.7 after autoclaving (Otrosoy, 2006). In some laboratories other substances, such as Jasmonic acid, NAA, Coumarine acetic acid, Absciscic acid and Triazole cytokinines (especially BA) were added to nutrient media to induce microtuber production (Khurana *et al.*, 2003).

Gibberellic acid can stimulate sprouting and advance emergence and break dormancy. Effects of gibberellic acid applied to the tuber on sprout growth include more sprouts, more branching of sprouts, thinner sprout and longer sprouts (Otrosoy, 2006).

There are many more growth regulators which can be applied to affect seed tuber dormancy or growth vigour of tubers. These include rindite (a mixture of ethylene chlorhydrin, ethylene dichloride and carbon tetrachloride), ethylene chlorhydrin, and carbon disulphide, thiourea and benzyl adenine (Struik and Wiersema, 1999).

Microtubers can be produced in *in vitro* conditions under dark condition or light conditions both with a temperature of 24 °C but microtuber production can be better in light (a short day of 8 h) than in dark (Ranalli, 1997). According to Pruski (2003) better results for microtuber production (higher number of uniform microtubers with larger size and higher weight) can be obtained under light exposure (8 h) than under

conditions without light, and also on solid medium than on liquid medium. (Otroty, 2006) reported that tuberization can be promoted with low temperature and short days; short days (8 hours of light) and low night temperatures promotes yields of tuber while no tuber is formed under long days (16 hours or longer) and high night temperatures. At low night temperature a tuber inducing hormone can be produced in the leaves which are then translocated to the stolon tip.

Extensive physiological research has revealed that tuberization is controlled by several factors. Tuberization initiation is also associated with hormonal changes in the plant (Vreugdenhil & Struik, 1989). Several workers have focused their attention on the application of exogenous growth regulators for stimulating *in vitro* tuberization (Coleman *et al.*, 2001). Growth regulators are commonly used in *in vitro* multiplication, both during the production of plantlets and in microtuber production. Coumarin is highly effective as a tuberization promoter (Radouani, 1997). A concentration of 25 mg l⁻¹ was optimum for tuberization stimulation and the optimum temperature for coumarin activity is between 20 to 25°C (Panis *et al.*, 2005). Coumarin is also affected by the concentration of sugar in the nutrient medium. Activity is reduced if the percentage of sugar is lower than 4% or higher than 10% (Roca *et al.*, 1978).

Sucrose has been established as prime component for potato micropropagation (Khuri and Moorby, 1995). There have been several reports comparing the effects on micropropagation of a range of sucrose concentrations and established that 3% sucrose was the optimum level for *in vitro* potato micropropagation (Pruski *et al.*, 2003). Sucrose is frequently used as a carbon source in plant tissue culture media. Its hydrolysis into glucose and fructose has been demonstrated in a wide variety of plant cell and tissue cultures (Mroginski *et al.*, 2007). Akita and Takayama, (1994) also reported that in their potato microtuber, all the remaining sugars were glucose and fructose after 10 weeks of *in vitro* growth *i.e.* total sucrose degraded into glucose and fructose. Such a hydrolysis of sucrose makes its utilization as the superior carbon source in potato micropropagation very insufficient (Yu *et al.*, 2000).

Conventional method of vegetative propagation in potatoes are often prone to pathogens such as fungi, bacteria and viruses, thereby poor quality and yields are obtained low. Therefore healthy potato seed should be used for regeneration every

tion has been focused on the in vitro culture for (Keller *et al.*, 2006).

production has become popular world wide.

size range of 5 – 20 mm in diameter or often even

in involves two stages: 1) *in vitro* multiplication

2) production of minitubers in the greenhouse.

minitubers, including minituber production in

uction on solid media. Both ways can involve

most common and practical way to produce

in vitro plantlets and plant them in normal potting soil in the

house, glasshouse or screenhouse (Struik & Wiersema, 1999). In this way, per in

vitro plantlet two to more than ten minitubers can be produced (Struik & Wiersema,

1999). Many factors operating during the second stage, including variety, size of

containers, growth regulators, plant density etc., can affect minituber yields. Not only

yield, but also size distribution of minitubers is important, as this affects the growth

vigour, number of stems of the plants grown from them, and number of tubers per

stem, and thus their commercial value in different stages of the seed tuber production

scheme (Shibli *et al.*, 2004).

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

The experiment was carried out at the Plant Tissue Culture Laboratory of Agrotechnology Discipline, Khulna University, Khulna-9208, Bangladesh, during December 2009 to January 2011.

3.1. Materials

Potato variety: Laura, Natura and Smart.



Variety: Laura



Variety: Natura



Variety: Smart

Source of the variety: The Potato varieties were collected from Potato Research Centre, Bangladesh Agriculture Research Institute (BARI), Gazipur, Dhaka.

Type of explants: Pre-established potato sprouts were used as source material for meristem culture.

Microscope used: Stereoscopic microscope, Model: Labomed CZM6.

Choice of culture media

For all the in vitro studies, MS (Murashige and Skoog, 1962) medium supplemented with 8 g l⁻¹ agar, 30 g l⁻¹ sucrose and different combinations and concentrations of growth regulators were selected.

3.2. Methods

3.2.1. Preparation of stock solutions for MS medium

During the preparation of culture media various ingredients were needed for the medium. For macronutrients, micronutrients, vitamins, growth hormones etc. preparation as separate stock solutions different ingredients were required in different concentrations.

3.2.1.1. Macro nutrient stock

One liter (1000 ml) of distilled water, ten times the weight of salts required per liter of the medium were weighed accurately and dissolved one by another in 750 ml of distilled water and the final volume was made up to 1000 ml by further addition of distilled water. This stock solution was filtered and poured into a conical flask. This was made up to 10 folds (10 x) of the final strengths of the medium in bottle with label and stored in a refrigerator at 4⁰ C for later use.

3.2.1.2. Micro nutrient stock

The stock solution of micronutrients was made up to 100 folds (100 x) of the final strength of the medium in 100 ml of distilled water. The stock solution was filtered, labeled and stored in refrigerator at 4⁰ C for later use.

3.2.1.3. Iron stock

This was prepared at 10 folds (10 x) of the final strength of FeSO₄ and Na₂-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 poured into clean amber glass bottle and stored in a refrigerator at 4⁰C.

3.2.1.4. Vitamin stocks

Each of the desired ingredients except Myo-inositol were taken at 100 folds (100 x) of their final strength in a measuring cylinder and dissolved in 150 ml of distilled water.

Then the final volume was made up to 100 ml by further addition of distilled water. Myo-inositol was prepared individually as above mentioned procedure. All the vitamins stocks were stored below 0°C.

3.2.2. Growth hormone [Gibberellic acid (GA₃), Napthalene acetic acid (NAA) and Benzyl amino purine (BAP)] stocks

To prepare the stock solution of GA₃, NAA and BAP 30 mg of each hormone was placed individually on clean 50 ml beaker and then dissolved in 1 ml of pure ethanol. The mixture was then taken into 100 ml conical flask and by adding distilled water the volume was made up to 100 ml. The solution was then stored at 4-6°C in refrigerator.

3.2.3. Preparation of culture media

Media were prepared with intensive care and the preparation procedure was as follows:

For one liter of agarified MS medium, total amount of agar powder (8 g) was added to the half volume of required distilled water (500 ml) in a beaker and dissolve the agar in micro oven. Total amount of sucrose (30 g l⁻¹) was dissolved in 250 ml double distilled water in another beaker. Required amount of macro, micronutrients and vitamins were mixed with the sucrose solution. Then the mixture was added to boiling agar solution and well stirred. This solution was taken in a volumetric flask, hormone stock was added and distilled water was added in the medium to make up the necessary volume (1 L). pH of the medium was adjusted between the range 5.6 to 5.8 by adding 0.1N HCl or 0.1N NaOH before autoclaving. Then the medium was poured into test tube and each test tube contained 25 ml medium. The test tube were closed with cotton plug and covered with brown paper and autoclaved at 121°C maintaining 1.5 kg cm⁻² pressures for 15 to 20 minutes.

3.2.4. Growth regulators used for meristem proliferation and multiplication of plantlets

To investigate the proliferation of meristems and subsequent plant regeneration, meristems were cultured on solidified MS medium supplemented with different combinations of BAP, NAA and GA₃. Each treatment consisted of 10 replications.

Table 1. Combinations and concentrations of growth regulators used for meristem proliferation treatments.

Treatment	GA ₃ (mg l ⁻¹)	BAP (mg l ⁻¹)	NAA (mg l ⁻¹)
1	2	0.5	0.5
2	3	0.5	0.5
3	4	0.5	0.5
4	5	0.5	0.5
5	6	0.5	0.5
6	2	1.0	0.5
7	3	1.0	0.5
8	4	1.0	0.5
9	5	1.0	0.5
10	6	1.0	0.5

For multiplication of regenerated plants, different levels of GA₃ in combination with NAA were used.

Table 2. Different levels of GA₃ in combination with NAA (0.1 mg l⁻¹) used for multiplication of regenerated plants.

Treatment	GA ₃ (mg l ⁻¹)	NAA (mg l ⁻¹)
1	1	0.1
2	2	0.1
3	3	0.1
4	4	0.1
5	5	0.1

GA₃ (5 mg l⁻¹) along with NAA (0.1 mg l⁻¹) supplemented with MS medium were also used to observe the performance of plantlets obtained from apical and axillary buds. Axillary bud-1, axillary bud-2, axillary bud-3 and axillary bud-4 were used in this study.

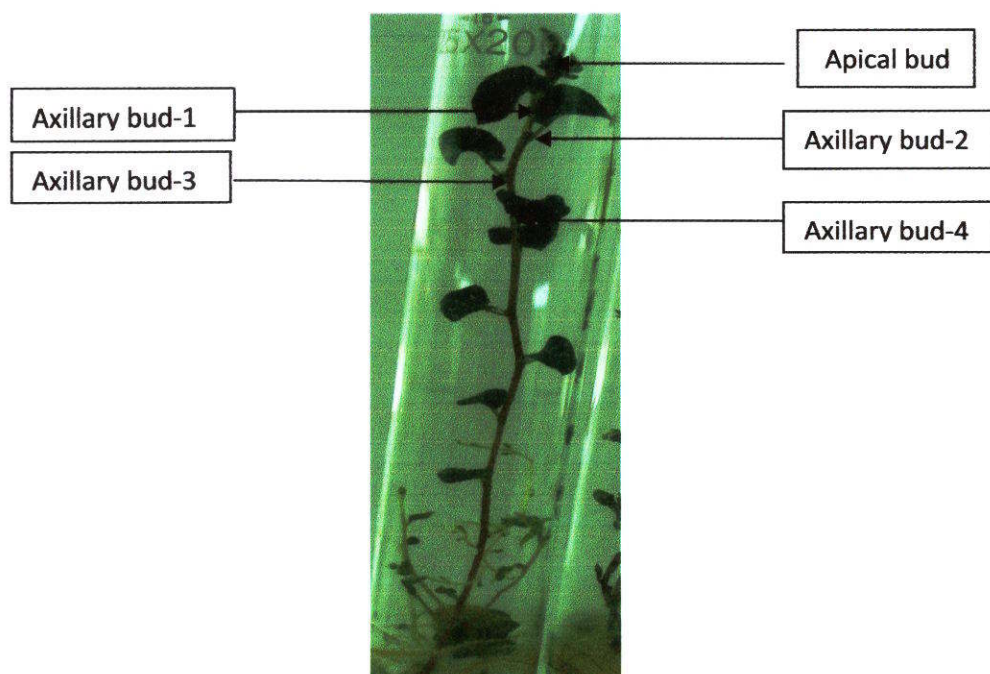


Fig 1. Plant indicating apical and axillary buds.

3.2.5. Constituents of media for microtuberization of potato:

For microtuber formation, MS medium supplemented with BAP-5.0 mg l⁻¹, Chloro Choline Chloride (CCC)-500 mg l⁻¹ and different concentrations of sucrose-5%, 6%, 7%, 8%, 9%, 10% were used.

3.2.6. Substrates used in pots for minituber formation:

Following substrates were employed for minituber production:

- | | |
|----------------------------|-----------|
| 1. Soil | 100% |
| 2. Sand | 100% |
| 3. Coconut dust | 100% |
| 4. Soil : Sand | 50% : 50% |
| 5. Soil : Coconut dust | 25% : 75% |
| 6. Soil : Coconut dust | 50% : 50% |
| 7. Soil : Coconut dust | 75% :25% |
| 8. Directly into the field | |

3.2.7. Sterilization

In *in vitro* techniques, aseptic condition is the essential task. So, all the instruments, glass wares and culture media were sterilized carefully.

3.2.7.1. Sterilization of glass wares and instruments

Beakers, test tubes, conical flasks, pipettes, were sterilized in an autoclave at a temperature of 121⁰C for more than 30 minutes under 1.5 kg cm⁻² pressures. Metal instruments viz. forceps, scalpels, needles, spatulas etc. were covered with aluminum foils and were sterilized by dry heat sterilization and also were sterilized by dipping them in absolute ethanol followed by flaming.

3.2.7.2. Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all floors and fumigated with formaldehyde. The process of sterilization was repeated at regular intervals. Generally, laminar airflow cabinet was sterilized by wiping the internal surface with pure ethyl alcohol every time before use.

3.2.8. Experimental design

All the experiments were laid out in a Completely Randomized Design (CRD). First three experiments had ten replication and microtuberization, fifteen replications were used. A test tube (15 cm length) containing 15 ml medium with a single shoot tip of potato was considered as a replication. For the experiment of minituber production, five replications for each treatment were taken. In that case, a plastic pot containing different substrates with a single healthy plant was considered as a replication.

3.2.9. Procedure for meristem culture establishment

Well-developed, healthy sprouts measuring 2 cm in length grown in laboratory condition were cut from the tubers. Then they were washed thoroughly with tap water and transferred to the laminar airflow cabinet. The cut sprouts were surface sterilized by dipping them in 70% ethanol for 30 seconds and immediately washed with sterilized distilled water. After washing, the sprouts were kept in 0.1 percent aqueous solution of HgCl_2 for 10 minutes. Then the sterilized sprouts were washed 3 times with sterilized double distilled water to remove trace of HgCl_2 . The surface water of treated materials were then dried with the autoclaved tissue paper and kept for few minutes in laminar airflow on an open Petridis for final drying.

Under a binocular dissecting microscope, leaflets surrounding the growth point are removed, until only the apical bud's dome with a few primordium leaves remain (usually two).



Fig. 2. Potato sprouts



Fig. 3. Excision of meristems under a binocular dissecting microscope.

3.2.9.1. Precautions to ensure aseptic condition

All inoculation and aseptic manipulations were carried out under a laminar airflow cabinet. The cabinet was switched on for half an hour before use, and then cleaned with pure ethanol to reduce the chances of contamination. The appliances like scalpels, forceps, needles, etc. were also cleaned and sterilized by dipping in 100% ethyl alcohol followed by flaming and cooling inside the inoculation chamber before use. Hands were rubbed with pure ethyl alcohol for sterilization. Aseptic conditions were followed during each and every operation to avoid contamination.

3.2.9.2. Inoculation of explants

Personal hygiene is also important in tissue culture. Hands were carefully washed by 70% ethanol before inoculation. Forceps and scalpels were dipped in absolute ethanol and flamed until red-hot. They were allowed to cool and then used for inoculation. Meristem tips were isolated from the sprouts, by a sterilized sharp scalpel under a binocular dissection microscope were transferred onto the nutrient media, after opening the cap the neck of the culture vessel was flamed, then after inoculation of the explants inside the culture vessel, its neck was flamed again before closing the cap near the flame to prevent the risk of contamination.

3.2.9.3. Incubation of the cultures

After inoculation of the explants, culture vessels were kept in a growth chamber with 16 hours photoperiod at about 2000 lux and at a temperature of $25 \pm 1^\circ\text{C}$ and relative humidity 65%. Proliferation of the meristem tip was first observed at 38 days after inoculation.

3.2. 10. Procedure for multiplication of regenerated plants

After taking all the precautions to ensure aseptic condition, *in vitro* grown plantlets were selected for regeneration. All inoculation and aseptic manipulations were carried out under a laminar airflow cabinet. Plantlets were removed carefully and cut into 1 to 2 cm long. Then they were placed onto nutrient media containing test tubes for further regeneration.

3.2.11. Procedure for multiplication of regenerated plants obtained from shoot buds of different positions in plant body

Plantlets were removed carefully and cut apical bud, axillary bud-1, axillary bud-2, axillary bud-3, axillary bud-4 into 1 to 2 cm long separately. Then they were placed onto nutrient media carefully.

3.2. 12. Procedure for microtuberization

After taking all the precautions to ensure aseptic condition, *in vitro* grown healthy plantlets were selected for further inoculation. All inoculation and aseptic manipulations were carried out under a laminar airflow cabinet. Plantlets were removed carefully and cut the shoot tips measuring 2 cm in length. Then they were placed onto nutrient media containing test tubes for microtuberization.

After inoculation of the explants, the microtuber induction cultures were incubated in the dark at 24°C and relative humidity 65%, maintaining a temperature of 18° C. Microtuber was first observed at 35 days after inoculation.

The experiment was laid out in a Completely Randomized Design (CRD) with ten replications. A test tube (15 cm length) containing 15 ml medium with a single shoot tip of potato was considered as a replication.

3.2. 13. Procedure for minituber production

For minituber production of potato, *in vitro* cultured plants were transplanted into plastic pots (4 inch size) with different substrates (soil, sand, coconut dust and their different combination) and some were directly into the field. Soil, sand and coconut dust were autoclaved at 121° C for 15 minutes before transplantation. After proper mixing of different substrates according to each treatment, they were kept in plastic pots. Few minutes later after watering, plants were transferred into pots. Transferred plants were placed into growth chamber with 85% humidity for 24 hours. After that, they were kept in net house.

3.2.14. Parameters studied and data collection

To study the survivability and proliferation of meristems,

- number of survived meristem tips,
- percentage of callus initiation,
- callus size,
- percentage of shoot initiation,
- number of shoot per plant,
- shoot length per plant and
- percentage of root initiation were recorded.

Data were collected three times after every 20 days interval.

For multiplication of regenerated plants,

- survival rate,
- shoot length per plant,
- branched/ unbranched and
- healthy/ unhealthy were also recorded.

Plants, whether they were branched or unbranched and healthy or not, were observed visually. Data were collected three times after every 10 days interval.

To study the performance of plantlets obtained from apical and axillary buds, data were collected by measuring

- percentage of survivability of plants,
- shoot length per plant,
- no. of branch per plant,
- root length per plant and
- Condition of shoots, whether they were healthy or not was observed visually.

Data were collected three times after every 10 days interval.

To study the effect of different sugar concentrations on *in vitro* microtuberization,

- survivability of plant,
- percentage of plant producing microtuber,
- no. of microtuber per plant and
- average diameter of tuber per plant were measured.

After 20 days interval, data were collected.

For experiment of *ex vitro* minituber production, data were collected by measuring

- percentage of survivability of plants up to 30 days,
- leaf color,
- shoot length per plant,
- no. of tuber per plant,
- average diameter of tuber per plant and
- average weight of tuber per plant.

Leaf color of every plant was observed visually.

3.2.15. Analysis of data

Recorded data for all of the experiments were analyzed for ANOVA (Analysis of Variance) with the help of computer using MSTAT-C program and the means were compared according to Duncan's Multiple Range Test (DMRT) and Least significance Differences (LSD).

CHAPTER FOUR

RESULTS AND DISCUSSION

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Results of the experiment

A series of *in vitro* as well as *ex vitro* experiments were conducted to fulfill the specific objectives. In this chapter, the results of the experiment have been presented, explained and discussed under different sub-headings.

4.1.1. Meristems response in culture

Meristems of three potato varieties were aseptically cultured on nutrient media, containing different combinations and concentrations of GA₃, BAP and NAA. Ten different combinations of these growth hormones were examined to find out their effects on meristem proliferation and plant regeneration; simultaneously the effect of varieties and variety X growth hormone interactions on meristem proliferation and further development were also investigated.

The results of effect of growth hormone have been shown in table 3. Significant role of growth hormones was noticed in callus induction, callus size and in root formation, however in shoot proliferation the role of growth hormone was found insignificant. Maximum callusing (83.33%) was observed in the medium containing 2 mg l⁻¹ GA₃, 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA followed by 70% callus in the medium fortified with 3 mg l⁻¹ GA₃, 1 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA. Root induction (40% and 30% respectively) was also significantly higher in these media. Shoot induction (3.33%) was occurred in the media containing 5 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA and 6 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA whereas, no. of branch per plant and average shoot length were higher in media containing 6 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA.

Table 3. Combined effect of growth regulators on meristem proliferation and further development.

Growth regulators (mg l ⁻¹)			Growth parameters of regenerated plants (Average plant ⁻¹)					
GA ₃	BAP	NAA	Frequency of meristem producing callus (%)	Average callus size (mm)	Frequency of meristems producing shoots (%)	No. of branches plant ⁻¹	Average shoot length (mm)	Frequency of meristems producing roots (%)
2	0.5	0.5	0e	0d	0	0	0	0b
3	0.5	0.5	0e	0d	0	0	0	0b
4	0.5	0.5	6.67 de	0.41 cd	0	0	0	10b
5	0.5	0.5	26.67 cde	1.86abc	3.33	0.03	0.38	6.67b
6	0.5	0.5	50bc	2.59ab	3.33	0.13	2.98	3.33b
2	1.0	0.5	83.33a	2.9a	0	0	0	40a
3	1.0	0.5	70ab	2.6ab	0	0	0	30a
4	1.0	0.5	30cd	1.12bcd	0	0	0	6.67b
5	1.0	0.5	43.33bc	1.81abc	0	0	0	10b
6	1.0	0.5	40c	1.51abcd	0	0	0	6.67b

Data in a column followed by same letters are not significantly different according to (DMRT).

Varietal effect on meristem proliferation has been presented in table 4. Regarding most of the parameters, the role of the potato varieties was insignificant except rooting, the variety responded well in rooting (20%) and 4% of the inoculated meristems of this variety produced shoots with an average shoot length of 1.48. It should also be mentioned that other two varieties viz. Natura and Laura did not respond at all to shoot formation from inoculated meristems.

Table 4. Effect of potato varieties on meristem proliferation and further development.

Variety	Growth parameters of regenerated plants (Average plant ⁻¹)					
	Frequency of meristem producing callus (%)	Average callus size (mm)	Frequency of meristems producing shoots (%)	No. of shoots plant ⁻¹	Average shoot length (mm)	Frequency of meristems producing roots (%)
Smart	36	1.54	4	0.08	1.48	20a
Natura	30	1.33	0	0	0	6b
Laura	39	1.57	0	0	0	8b

Data in a column followed by same letters are not significantly different according to DMRT.

Interaction effect of growth regulators X varieties on different parameters have been shown in the table 5. Data presented in the table show that only root formation was found significant. Maximum rooting (80%) was noticed in the variety Smart on the medium containing 2.0 mg l⁻¹ GA₃, 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA. Maximum callusing was observed in Smart variety on the medium 3.0 mg l⁻¹ GA₃, 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA. In the variety Natura and Laura on the medium containing 2.0 mg l⁻¹ GA₃, 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA, shoot proliferation over all was very poor, only the variety Smart responded in shooting on the media fortified with 5.0 and 6.0 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA.

Table 5. Effect of various combinations and concentrations of growth regulators on growth parameters of meristems of three potato varieties.

Variety	Growth regulators (mg l ⁻¹)			Growth parameters of regenerated plants (Average plant ⁻¹)					
	GA ₃	BAP	NAA	Frequency of meristem producing callus (%)	Average callus size (mm)	Frequency of meristems producing shoots (%)	No. of shoots plant ⁻¹	Average shoot length (mm)	Frequency of meristems producing roots (%)
Smart	2	0.5	0.5	0	0	0	0	0	0c
	3	0.5	0.5	0	0	0	0	0	0c
	4	0.5	0.5	20	1.23	0	0	0	30bc
	5	0.5	0.5	20	1.41	10	0.1	1.13	20bc
	6	0.5	0.5	30	1.97	10	0.4	8.95	0c
	2	1.0	0.5	80	3.12	0	0	0	80a
	3	1.0	0.5	100	3.24	0	0	0	20bc
	4	1.0	0.5	40	1.38	0	0	0	20bc
	5	1.0	0.5	40	1.84	0	0	0	10bc
	6	1.0	0.5	30	1.20	0	0	0	20bc
Natura	2	0.5	0.5	0	0	0	0	0	0c
	3	0.5	0.5	0	0	0	0	0	0c
	4	0.5	0.5	0	0	0	0	0	0c
	5	0.5	0.5	20	2.10	0	0	0	0c
	6	0.5	0.5	60	3.32	0	0	0	0c
	2	1.0	0.5	80	2.84	0	0	0	0c
	3	1.0	0.5	40	1.22	0	0	0	40b
	4	1.0	0.5	20	0.73	0	0	0	0c
	5	1.0	0.5	40	1.81	0	0	0	20bc
	6	1.0	0.5	40	1.29	0	0	0	0c
Laura	2	0.5	0.5	0	0	0	0	0	0c
	3	0.5	0.5	0	0	0	0	0	0c
	4	0.5	0.5	0	0	0	0	0	0c
	5	0.5	0.5	40	2.08	0	0	0	0c
	6	0.5	0.5	60	2.48	0	0	0	10bc
	2	1.0	0.5	90	2.74	0	0	0	40b
	3	1.0	0.5	70	3.36	0	0	0	30bc
	4	1.0	0.5	30	1.27	0	0	0	0c
	5	1.0	0.5	50	1.79	0	0	0	0c
	6	1.0	0.5	50	2.03	0	0	0	0c

Data in a column followed by same letters are not significantly different according to (DMRT).

After 11 weeks of meristem culture, it was noticed that shoots were formed only from Smart variety. Therefore, further regeneration was carried out with Smart variety. Plantlets formed in the variety Smart were multiplied clonally by shoot buds. In this experiment, different concentrations of GA₃ were examined. The results of this experiments are shown in the table 6. No significant results have been obtained from this study. In all the treatments, all parameters showed similar results. Although the medium fortified with 5 mg l⁻¹ GA₃ + 0.1 mg l⁻¹ NAA was found better in highest shoot length (91.45 mm) and root length (35.28 mm).

Table 6. Effect of different concentrations of GA₃ in combination with NAA on multiplication of regenerated plants of variety Smart.

Growth regulators (mg l ⁻¹)		Survivability (%)	Shoot length (mm)	Branching pattern	Condition of shoots	Root length(mm)
GA ₃	NAA					
1.0	0.1	100	30.73	Branched	+	22.14
2.0	0.1	100	43.97	Branched	+	28.71
3.0	0.1	100	78.62	Unbranched	+	26.88
4.0	0.1	100	45.5	Branched	++	19.46
5.0	0.1	100	91.45	Unbranched	+++	35.28
Level of significance		NS	NS			NS

+++ indicates healthy, ++ indicates moderate healthy, + indicates unhealthy

Shoot buds of different positions in plant body were also investigated for *in vitro* clonal multiplication. Significant results were obtained in most of the parameters. In all respects, apical buds showed better performance (Table 7). The highest shoot length was 64.26 mm observed in apical bud whereas, the lowest in axillary bud - 4. The highest root length (35.55 mm) and healthy plants were also found in apical bud (Table 7).

Table 7. Performance of plantlets obtained from apical and axillary buds.

Used explants	Survivability %	Shoot length (mm)	Average no. of branch plant ⁻¹	Condition of shoot	Root length (mm)
Apical bud	100	64.26	1.3	+++	35.55
Axillary bud -1	100	52.00	1.7	+++	29.74
Axillary bud - 2	100	37.33	1.3	++	28.26
Axillary bud -3	90	48.21	1.2	+	27.36
Axillary bud -4	70	36.31	0.8	++	21.71
LSD at 0.05	NS	17.03	0.56		NS

+++ indicates healthy, ++ indicates moderate healthy, + indicates unhealthy

4.1.2. Effect of different sucrose concentrations on *in vitro* microtuberization of potato

Shoots of apical portion measuring 2 cm in length were aseptically cut from *in vitro* grown potato plantlets were inoculated on media containing variable doses of sucrose. The media were also fortified with CCC at 500 mg l⁻¹ and BAP at 5 mg l⁻¹. The results of the experiments (Table 8) revealed that the percentage of plants producing microtuber increased with the increase of sucrose concentrations. Percentage of survivability also increased with the increase of sucrose concentration. The highest survival rate (90%) was found at 10% sucrose. The highest number of microtuber was 2.17 observed on 8% sucrose where as the lowest number was 1.5 at 6% sucrose. In case of average diameter of tuber plant⁻¹, it was observed that 10% sucrose showed best result. Average diameter of tubers varied from 3.12 mm to 5.11 mm irrespective of sucrose concentrations.

Table 8. Effect of different sucrose concentration in presence of BAP (5 mg l⁻¹) and CCC (500 mg l⁻¹) on *in vitro* microtuberization from cultured shoot tips.

Sucrose concentration (%)	Survivability (%)	Plants producing microtuber (%)	No. of microtuber plant ⁻¹	Average diameter of tuber plant ⁻¹ (mm)
5	40	25	2	4.62
6	40	50	1.5	3.12
7	50	40	2	4.44
8	60	90	2.17	3.91
9	60	90	1.33	4.5
10	90	100	2	5.11
Level of significance	NS	NS	0.01	0.01
LSD	-	-	1.363	2.285

4.1.3. Effect of different substrates on *Ex vitro* performance of plants and minituber production of potato

In vitro grown potato plantlets were carefully removed, washed and planted into different substrates to investigate *ex vitro* performance of plants and performance of minituber production. Survival rate of plants after 30 days was found similar for all substrates whereas, leaf colors of plants varied from substrates to substrates. Minituber production on direct field showed best performance. It was observed that after 30 days, maximum shoot length plant⁻¹ (188.87 mm) was found on the direct field whereas the lowest shoot length plant⁻¹ (69.32 mm) was on sand. Average weight of tuber plant⁻¹ varied from 0.77 gm to 6.17 gm irrespective of different substrates. Lowest weight of tuber plant⁻¹ was recorded on the substrates containing coconut dust; although soil, sand, coconut dust, 50% soil + 50% sand, 25% soil+ 75%

coconut dust, 50% soil + 50% coconut dust and 75% soil + 25% coconut dust were found statistically similar effective for weight of tuber. The highest weight of tuber was found in direct field. In case of, Average diameter of tuber plant⁻¹, 25% soil+ 75% coconut dust showed minimum result and on the other hand, in direct field, large size of tubers were found (Table 9).

Table 9. *Ex vitro* minituber production using different substrates.

Substrates	Survivability (%)	Leaf color	Shoot length(mm)	No. of tuber plant ⁻¹	Average diameter of tuber plant ⁻¹ (mm)	Average weight of tuber plant ⁻¹ (gm)
Soil	100	Green	131.76b	3.4bc	15.20	2.47b
Sand	100	Yellowish	66.81c	2.0bc	15.09	1.23b
Coconut dust	100	Yellowish	69.32c	1.0c	12.94	0.77b
50% soil + 50% sand	100	Light green	95.85bc	2.75bc	14.85	1.5b
25% soil + 75% coconut dust	100	Yellowish	92.14bc	4.6bc	12.49	1.36b
50% soil + 50% coconut dust	100	Light green	117.4b	4.5bc	12.53	1.63b
75% soil + 25% coconut dust	100	Green	135.34b	5.6b	14.37	2.6b
Direct on field	100	Dark green	188.87a	9.4a	15.5	6.17a
Level of significance	NS		0.01	0.01	NS	0.01

Data in a column followed by same letters are not significantly different according to DMRT.

4.2. Discussion

In the present investigation, various concentrations of two growth hormone viz. GA₃ and BAP in combination with NAA were tested for the proliferation of potato meristems and for their further growth and development. Varietal performance in this regard was also studied.

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (Gaur, 1999). Chandra (1994) developed a suitable MS medium supplemented with 1.0 mg l⁻¹ BAP, 0.01 mg l⁻¹ NAA and 1.0 mg l⁻¹ GA₃. BAP is an essential exterior plant growth regulator in potato shoot tip culture. However, a high concentration easily induced multiple shoot but have stunted growth, so a low concentration (0.5–1.0 mg l⁻¹) should be used (Otroshy, 2006). This experiment agrees with the previous study that variation in concentration and combination of BAP, NAA and GA₃ showed growth and morphological changes. BAP concentration (0.5 mg l⁻¹) was best shoot induction plant growth regulator for Smart variety.

Although higher concentration of GA₃ in combinations with BAP and NAA showed poor performance in regenerating shoot tips from meristem but overall its performance was better. Some authors reported similar trends for application of GA₃ (Ahsan *et al.*, 2003).

In this study of Shoot Multiplication of regenerated plants, the effect of gibberellin in combination with auxin were compared. In general, the results demonstrated that the most healthy plants and highest mean of shoot length per explants was obtained from MS medium containing 5 mg⁻¹ GA₃ and 0.1 mg⁻¹ NAA (Table 6). This may be due to the effect of gibberellin. It promotes the shoot proliferation (Hawkes, 1990).

In this investigation, result showed that apical buds represented best result than axillary buds. The differential response of apical and axillary buds may be due to the difference in morphogenetic potential and their inherent capabilities. Likewise, Pruski, (2007) and Beukema *et al.*, (1990) reported that the choice of an appropriate explant is critical for success in morphogenesis and the ability of various types of

tissues to respond for regeneration depends on their inherent capabilities. Moreover, Huang *et al.*, (1995) compared the *in vitro* reactivity of explants of black locust from crown branches with basal sprouts of the same mature tree. They concluded that shoots derived from the top branches showed more rapid growth than the shoots derived from epicormic branches. Differential meristematic activity within different plant parts with respect to position, may also suggest the different regeneration potential of the buds. It is probable that the better response of apical buds may be due to higher meristematic activity as compared to axillary buds. In this respect, Radouani, (1997) stated that meristematic activity in meristem of apical buds is greater than axillary buds. Further, it may be hypothesized that accumulation of different inhibitors may occur in axillary buds leading to reduced length. Suzuki *et al.*, (2006) observed that buds situated lower on mulberry shoots are more inhibited as compared to the buds present on the top. He detected abscisic acid and phenolic compounds accumulation in the axillary buds and attributed the growth inhibition to these inhibitors; which generally induce a shortening of the internode of higher plants *In vivo* (Hirai, 1999). Furthermore, Midmore (1992) reported that phenolic compounds frequently develop browning and are well known to be inhibitory to plant's cellular growth.

Normal (non *in vitro*) tuberization of potato is induced by short days, high light intensity, low night temperature, low nitrogen and physiological old tubers. When the plant is in induced condition, tuberization is not restricted to underground stem (stolon) but every axil of the plant can produce tubers. The endogenous cytokinin, gibberellin like substance, and starch are different between the inducing and non inducing tissue. During the stolon formation (non induced) the endogenous gibberellin like substance are high but the endogenous cytokinin and starch are low. The reverse are true prior to tuberization and at tuberization.

Sucrose has been established as prime component for potato micropropagation (Khuri and Moorby, 1995). There have been several reports comparing the effects on micropropagation of a range of sucrose concentrations and established that 3% sucrose was the optimum level for *in vitro* potato micropropagation (Pruski *et al.*, 2003). For optimal plantlet growth, sucrose level sustainability are necessary and if it

is rapidly hydrolyzed into glucose and fructose making the long term maintenance of desirable sucrose level is difficult. The present investigation was tended to find out the effect of sugar level on microtuberization and found that microtuberization increased with the increasing sugar level and the optimum concentration was 10% sucrose which was dissimilar to Pruski *et al.*, (2003).

In *in vivo* condition average tuber weight and tuber number were determined as different depending on the production technique (Leifert and Struik, 1992; 1999). Keller *et al.*, (2006) found that the tuber numbers of various potato cultivars were between 6.2 to 7.9 in plantlets grown in polyethylene bags. According to Wang *et al.*, (1982) also some soil-less production systems have been developed for minituber production: minitubers can also be produced through hydroponic systems (soil-less systems). Less risk of infection by different pathogens, high rate of multiplication and higher number of minitubers per unit area are reported as advantages of the soil-less system (hydroponic system) for minituber production compared to minituber production in the soil such as peat (Rosell *et al.*, 1987). Also Otrushy (2006) reported that total production and number of tubers were higher in hydroponic systems compared to traditional culture, meaning culture in a peat/sand mixture. In this study of minituber production, result showed that plants which were directly transplanted into field represented best result than other substrates.

CHAPTER FIVE

SUMMARY AND CONCLUSION

CHAPTER FIVE

SUMMARY AND CONCLUSION

The present experiment was carried out at the Plant Tissue Culture Laboratory of Agrotechnology Discipline, Khulna University, Khulna. Sprouts of the three potato cultivars viz. Smart, Natura and Laura were used as source material for meristems. Well developed healthy sprouts measuring 2 cm in length grown in laboratory condition were taken from the tubers. Then they were washed with tap water and surface sterilized under laminar air flow cabinet. Under a binocular dissecting microscope, leaflets surrounding the growth point of sprouts were removed, until only the apical bud's dome and a few, usually two, primordial leaves remained.

The medium used was supplemented with various concentrations and combinations of growth regulators such as BAP, GA₃ and NAA. The effect of these growth regulators on callus formation and some growth parameters of regenerated plants of three potato varieties were analyzed. Different concentrations of GA₃ along with NAA were applied for choosing the suitable dose for multiplication of regenerated plants. Performance of plantlets obtained from different parts of plants were also studied.

MS media supplemented with various sucrose concentrations along with BAP and CCC were employed for microtuberization and for minituber production, different combination of soil, sand and coconut dust were used.

Variable results were obtained during the evaluation of growth parameters. After 30 to 40 days of culturing the meristems, various morphological changes were observed. This includes increasing meristem tissue volume, shoot and root development. Significant role of growth hormones was noticed in callus induction, callus size and in root formation, however in shoot proliferation the role of growth hormone was found insignificant. Maximum callusing (83.33%) was observed in the medium containing 2 mg l⁻¹ GA₃, 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA followed by 70% callus in the medium fortified with 3 mg l⁻¹ GA₃, 1 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA. Root induction (40% and 30% respectively) was also significantly higher in these media. Shoot induction (3.33%) was occurred in the media containing 5 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA and 6 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA whereas, no. of branch per plant and

average shoot length were higher in media containing $6 \text{ mg l}^{-1} \text{ GA}_3 + 0.5 \text{ mg l}^{-1} \text{ BAP} + 0.5 \text{ mg l}^{-1} \text{ NAA}$.

Regarding most of the parameters, the role of the potato varieties was insignificant except rooting, the Smart variety responded well in rooting (20%) and 4% of the inoculated meristems of this variety produced shoots with an average shoot length of 1.48 mm. It should also be mentioned that other two varieties viz. Natura and Laura did not respond at all to shoot formation from inoculated meristems.

Through the interaction effect of growth hormones X varieties, result showed that only root formation was found significant. Maximum rooting (80%) was noticed in the variety Smart on the medium containing GA_3 - 2.0, BAP- 1.0 and NAA- 0.5 mg l^{-1} . Maximum callusing was observed in Smart variety on the medium GA_3 - 3.0, BAP- 1.0 and NAA- 0.5 mg l^{-1} . In the variety Natura and Laura, maximum callusing was observed on the medium containing GA_3 -2.0, BAP- 1.0 and NAA- 0.5 mg l^{-1} , shoot proliferation over all was very poor, only the variety Smart responded in shooting on the media fortified with GA_3 5.0 and $6.0 \text{ mg l}^{-1} + \text{BAP- } 0.5 \text{ mg l}^{-1} + \text{NAA- } 0.5 \text{ mg l}^{-1}$.

After 11 weeks of meristem culture, it was noticed that shoots were formed only from Smart variety. Therefore, further regeneration was carried out with Smart variety. Plantlets formed in the variety Smart were multiplied clonally by shoot buds. In this experiment, different concentrations of GA_3 were examined. No significant results have been obtained from this study. In all the treatments, all parameters show similar results. Although the medium fortified with $5 \text{ mg l}^{-1} \text{ GA}_3 + 0.1 \text{ mg l}^{-1} \text{ NAA}$ was found better in highest shoot length (91.45 mm) and root length (35.28 mm).

Shoot buds of different positions in plant body were also investigated for *in vitro* clonal multiplication. Significant results were obtained in most of the parameters. In all respects, apical buds show better performance. The highest shoot length was 64.26 mm observed in apical bud whereas, the lowest in axillary bud - 4. Highest root length and healthy plants were also found in apical bud.

Percentage of survivability of microtuber producing plants also increased with the increase of sucrose concentration. Best survival rate was found at 10% sucrose. The highest number of microtuber was 2.17 observed on 8% sucrose where as the lowest number was 1.5 at 6% sucrose. In case of average diameter of tuber per plant, it was

observed that 10% sucrose showed best result. Average diameter of tubers varied from 3.12 mm to 5.11 mm irrespective of sucrose concentrations.

Minituber production on direct field showed best performance. It was observed that after 30 days, maximum shoot length plant⁻¹ (188.87 mm) was found on the direct field whereas the lowest shoot length plant⁻¹ (69.32 mm) was on sand. Average weight of tuber plant⁻¹ varied from 0.77 gm to 6.17 gm irrespective of different substrates. Lowest weight of tuber plant⁻¹ was recorded on the substrates containing coconut dust; although soil, sand, coconut dust, 50% soil + 50% sand, 25% soil+ 75% coconut dust, 50% soil + 50% coconut dust and 75% soil + 25% coconut dust were found statistically similar effective for weight of tuber. The highest weight of tuber was found in direct field. In case of, average diameter of tuber plant⁻¹, 25% soil+ 75% coconut dust showed minimum result and on the other hand, in direct field, large size of tubers were found.

The present study represents that meristems isolated from shoot tips of potato (*Solanum tuberosum* L.) cv. Smart carry a high potential for shoot and root formation on MS culture media supplemented with different concentrations and combinations of growth regulators. It will help the producers supplying with virus free uniform planting material by rapid multiplication and open commercial opportunities for the farmers.

REFERENCES

CHAPTER SIX

REFERENCES

- Abbott, A.J. and Belcher, A.R. (1986). Potato tuber formation *in vitro*. In: Plant tissue culture and its agricultural application. Withers, L.A. and Alderson, P.G.(Eds.), London: Butterworths, pp: 113-121.
- Aburkhes, M., N. Fahmi, A. Benhemida, M. Nafali and A. Zeiglem 1984. Virus free potatoes by tissue culture in Libya. *Acta horticulture* 289: 77-79.
- Ahsan, N., Hossain, A., Alam, M.F., Hossain, M.M., Islam, R. and Sultana, R.S. (2003). Virus-free potato tuber seed production through meristem culture in Tropical Asia. *Asian Journal of Plant Sciences* 2(8): 616-622.
- Akita, M., Takayama, S., (1994). Induction and development of potato tubers in a jar fermentar. *Plant Cell Tissue Organ Culture* 36: 177-182.
- Anonymous. 1992. Potatoes: Improving disease resistance and quality. *Biotechnology and Development Monitor*. 12: 3-5.
- Badoni, A. and Chauhan , J. S. 2009. Effect of growth regulators on meristem tip development and *in vitro* multiplication of potato cultivar “Kufri Himalini”. *Nature and science*, 7(9):31-34. (<http://www.sciencepub.net/nature>., accessed on 27th April , 2010).
- Bajaj, Y.P.S. 1981. Regeneration of plants from potato meristems freeze-preserved for 24 months. *Euphytica* 30:141-145.
- Behnke, M. 1993. Selection of potato callus for resistance to culture filtrates of *Phytophthora infestans* and regeneration of resistant plant. *TAG Theoretical and Applied Genetics*, 55(2): 69-71.
- Beukema, H.P. and D.E. Van Der Zaag, 1990. *Introduction to Potato Production*, p: 208. Pudoc. Wageningen Netherlands.
- Chandra, R. and R. K. Bihman 1994. *In vitro* micro propagation in relation to pedigree in potato. *Journal of Indian Potato Association*. 21:87
- Chawla, H.S. 2002. *Introduction to Plant Biotechnology*. Oxford & IBH Publising Co. Pvt. Ltd., New Delhi, pp. 41-56.
- Coleman, K.W., J.D. Danielle and S.E. Coleman, 2001. Potato micro-tubers as Research Tools: A Review. *American J. Potato Res.*, 78: 47–55

- Danielle, J.D., Warren, K.C. and Shirlyn, E.C. (2003). Potato microtuber production and performance: A Review. *American Journal of Potato Research* 80: 103-115.
- Dumet, D. and E.E. Benson. 2000. The use of physical and biochemical studies to elucidate and reduce cryopreservation-induced damage in hydrated/desiccated plant germplasm. In: Engelmann, F. and Takagi, H. (eds) *Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application*, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 43-56.
- Dumet, D., Y. Chang, B.M. Reed and E.E. Benson. 2000. Replacement of cold acclimation with high sucrose pretreatment in black currant cryopreservation. In: Engelmann, F. and Takagi, H. (eds) *Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application*, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 385-387.
- FAO. 2007. <http://www.potato2008.org/en/potato/>. Access date: 8th May 2008.
- FAOSTAT. 2007. <http://faostat.fao.org>. Access date: 8th May 2008.
- Frickey, T. and A.N. Lupas. 2004. Phylogenetic analysis of AAA proteins. *Journal of Structural Biology* 146:2-10.
- Gaur PC, Naik PC, Kaushik SK, Chakrabati SK. 1999. Indian Potato Varieties, CPRI Technical Bulletin, 51, 5-10
- Gebre, E. and Sathyanarayana, B. N. 2001. *In vitro* shoot regeneration and micro tuber production . *African Crop Science Journal*, 9(1): 1-8.
- Gopal, J., J. L. Minocha and H. S., Dhaliwal 1980. Microtuberization in potato (*Solanum tuberosum* L.). *Plt. Cell. Rep.* 17: 794-798.
- Griffiths, H.M., S.A. Slack and J.H. Dodds. 1990. Effect of chemical and heat therapy on virus concentration in *in vitro* plantlets. *Can. J. Bot.* 68:1515–1521.
- Halmagyi, A., C. Deliu and A. Coste. 2005. Plant regrowth from potato shoot tips cryopreserved by a combined vitrification-droplet method. *CryoLetters* 26:313-322.
- Hawkes, J.G. (ed) 1990. *The Potato: Evolution, Biodiversity and Genetic Resources*. Belhaven Press, London. 259.
- Hils, U. and L. Pieterse (eds) 2005. *World Catalogue of Potato Varieties 2005*. AGRIMEDIA, Bergen/Dumme, Germany. 243.

- Hirai, D. and A. Sakai. 1999. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. *Potato Research* 42:153-160.
- Hoque, M. I., Islam, M. A., Sarker, R.H. and Islam, A.S. 2003. In vitro microtuber formation in potato (*Solanum tuberosum* L.). *Nature and Science*, 6(9):31- 34.
- <http://www.potato2008.org/en/aboutiyp/index.html> ., accessed on 27th March 2010.
- <http://www.potato2008.org/en/world/asia.html> ., accessed on 27th March 2010.
- <http://scialert.net/abstract/?doi=ijv.2009.64.76>., accessed on 29th March, 2010.
- http://aob.oxfordjournals.org/cgi/pdf_extract/42/5/1227., accessed on 1st July, 2010.
- Huang, C.N., J.H. Wang, Q.S. Yan, X.Q. Zhang and Q.F. Yan. 1995. Plant-regeneration from rice (*Oryza-sativa* L.) embryogenic suspension cells cryopreserved by vitrification. *Plant Cell Reports* 14:730-734.
- International Potato Center (CIP). 1993. Basic techniques in plant virology. U. Jayasinghe y L.F. Salazar (eds.). CIP, Lima (Technical Training Unit 1).IPK. 2007. Annual Report.
- Keller, E.R.J., A. Senula, S. Leunufna, and M. Gröhe. 2006. Slow growth storage and cryopreservation - tools to facilitate germplasm maintenance of vegetatively propagated crops in living plant collections. *International Journal of Refrigeration* 29:411-417.
- Khan, S. A. K. U. and Rabbani, M. G. 1999. Effect of preincubation of tubers and on in vitro culture of three potato varieties. *Plant Tissue Culture*, 9(1):1-9.
- Khurana, S. M. P., Minhas, J.S. and Pandey, S. K. 2003. The potato production and utilization in sub tropics. Mehta publishers, New Delhi, India, 445 pp.
- Khuri, S. and Moorby, J. 1995. Investigations into the roles of sucrose in potato cv. Estima microtuber production *in vitro*. *Annals of Botany* 75: 295-303..
- Leifert, C., Pryce, S., Lumsden, P.J. and Waites, W.M. (1992). Effect of medium acidity on growth and rooting of different plant species growing *in vitro*. *Plant Cell Tissue and Organ Culture* 30: 171-179.
- Lizarrage, R. E., Tovar, P. and Jayasingha, U. 1986. Tissue culture for elimination of pathogens. Lima. pp.21.

KU.WO - ALL No - 3046 - ① sent 21.5.18

- Mahmoud, S. Y. M., Hosseiny, M. H. and Mamdouh, H. A. 2009. Evaluation of some therapies to eliminate potato Y potyvirus from potato plants. *Int. J. Virol.*, 5: 64-76.
- Midmore DJ (1992) Potato production in the tropics. In: *The potato crop, The scientific basis for improvement*, Harris PM (Ed.), Chapman and Hall, London, pp.728-793.
- Mroginski, L. and H. Rey. 2007. Cryopreservation of plant germplasm in Argentina. *Advances in Horticultural Science* 21:270-273.
- Otroshy, Mahmoud, 2006. Utilization of tissue culture techniques in a seed potato tuber production scheme. Doctorial thesis, Wageningen university, Wageningen, The Netherlands, pp. 17-24.
- Panis, B. and M. Lambardi. 2005. Status of cryopreservation technologies in plants (crops and forest trees). The role of biotechnology. Villa Gualino, Turin, Italy 5.-7.March 2005:43-54.
- Pennazio, S., and M. Vecchiati 1976. Effect of naphthalene acetic acid on meristem tips development. *Potato Research*, 19(3): 232-234
- Pruski, K. (2007). The Canon of Potato Science: *In vitro* multiplication through nodal cuttings potato research 50:293–296.
- Pruski, K., Astatkie, T., Duplessis, P., Lewis, T., Nowak, J. and Struik, P.C. 2003. Use of jasmonate for conditioning of potato plantlets and microtubers in greenhouse production of minitubers. *American Journal of Potato Research* 80: 183-193.
- Radouani, A., 1997. *In vitro* tuberization of potato cultivars, Nicola and Russet Burbank, as influenced by growth regulators, temperature and agitation. M.S. Thesis, University of Minnesota, St. Paul.
- Ranalli, P., 1997. Innovative propagation methods on seed tuber multiplication programmes. *Potato Res.*, 40: 439–53
- Reed, B.M. and P. Tanprasert. 1995. Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature. *Plant Tissue Culture and Biotechnology* 1:137–142.
- Roca, W.M., N.O. Espinosa, M.R. Roca and J.E. Bryan, 1978. A tissue culture method for the rapid propagation of potatoes. *American Potato J.*, 55: 691–701.

- Rosell, G., F. G. De Bestoldi and R. Tizio 1987. *In vitro* mass tuberization as a contribution to potato micro propagation. *Potato Research* 30(1): 111-116.
- Roset, S. and Bokelmann, G.S. 1996. Effect of growth regulators on *in vitro* multiplication of potato. Vegetative propagation of *Solanum tuberosum* L. in *in vitro* Potato, 19:173-8.
- Sarkar, D. and P.S. Naik. 1998. Cryopreservation of shoot tips of tetraploid potato (*Solanum tuberosum* L.) clones by vitrification. *Annals of Botany* 82:455-461.
- Schäfer-Menuhr, A. and H.M. Schumacher. 1997. Cryopreservation of potato cultivars - Design of a method for routine application in genebanks. *Acta Horticulturae* 447:477-482.
- Senula, A., E.R.J. Keller, T. Sanduijav and T. Yohannes. 2007. Cryopreservation of cold-acclimated mint (*Mentha spp.*) shoot tips using a simple vitrification protocol. *CryoLetters* 28:1-12.
- Seong, E.S., D. Choi, H.S. Cho, C.K. Lim, H.J. Cho and M.H. Wang. 2007. Characterization of a stress-responsive ankyrin repeat-containing zinc finger protein of *Capsicum annuum* (CaKR1). *Journal of Biochemistry and Molecular Biology* 40:952-958.
- Shibli, R.A., S.S. Al-Ababneh and M.A.L. Smith. 2004. Cryopreservation of plant germplasm: a review (scientific review). *Dirasat, Agricultural Sciences* 31:60-73.
- Spooner, D.M. and J.B. Bamberg. 1994. Potato genetic resources: sources of resistance and systematics. *American Potato Journal* 71:325-337.
- Struik, P.C. and S.G. Wiersema, 1999. *Seed Potato Technology*, p: 383. Wageningen Perss, Wageningen, The Netherlands
- Suzuki, M., M. Ishikawa, H. Okuda, K. Noda, T. Kishimoto, T. Nakamura, I. Ogiwara, I. Shimura and T. Akihama. 2006. Physiological changes in gentian axillary buds during two-step preculturing with sucrose that conferred high levels of tolerance to desiccation and cryopreservation. *Annals of Botany* 97:1073-1081.
- Taylor, M.J., Y.C. Song and K.G.M. Brockbank. 2004. Vitrification in tissue preservation: New developments. In: Fuller, B., Lane, N. and E.E. Benson (eds) *Life in the Frozen State*. CRC Press, London, New York. 603-644.

- Vandenbussche, B., S. Leuridan, V. Verdoodt, M. Gysemberg and M. De Proft. 1999. Changes in sugar content and fatty acid composition of *in vitro* sugar beet shoots after cold acclimation: influence on survival after cryopreservation. *Plant Growth Regulation* 28:157-163.
- Vreugdenhil, D. and P.C. Struik, 1989. An integrated view of the hormonal regulation of tuber formation in potato (*Solanum tuberosum*). *Physiol. Pl.*, 75: 525-31
- Wang, P. J. and C. V. Hu 1982. *In vitro* mass tuberization and virus free seed potato production in Tiwan. *Amer. Pot. Journ.* 59: 33-39.
- Ward, A.C.W., E.E. Benson, N.W. Blackhall, S. Cooperbland, W. Powell, J.B. Power and M.R. Davey. 1993. Flow-cytometric assessments of ploidy stability in cryopreserved dihaploid *Solanum tuberosum* and wild *Solanum* species. *CryoLetters* 14:145-152.
- Yoon, K.S. and Leung, W.M. (2004). Relative importance of maltose and sucrose supplied during a 2-step potato microtuberization process. *Acta Physiologiae Plantarum*. 26: 47-52.
- Yu, W.C., Joyce, P.J., Cameron, D.C. and Mc Cown, B.H. (2000). Sucrose utilization during potato microtuber growth in bioreactors. *Plant Cell Report* 19: 407-413.
- Zaman, M. S., Quraishi, A., Hassan, G., Raziuddin, Ali, S., Kabir, A. and Gul , N. 2001. Production of virus free plantlets. *On Line Journal of Biological Sciences*, 1(10): 898-899.
- Zhao, M.A., S.P. Dhital, Y.L. Fang, D.M. Khu, Y.S. Song, E.J. Park, C.W. Kang and H.T. Lim. 2005. Application of slow-freezing cryopreservation method for the conservation of diverse potato (*Solanum tuberosum* L.) genotypes. *Journal of Plant Biotechnology* 7:1-4.
- Ziv, M. and Shemesh, D. 2002. Propagation and tuberization of potato bud clusters from bioreactor culture. *Vitro Cell. Dev. Biol.-Plant*, 32:31-36.

APPENDICES

APPENDICES

Appendix-I

Composition of MS media

Nutrients status	Nutrients	MS (mg l ⁻¹)
Macro	KNO ₃	1900
	NH ₄ NO ₃	1650
	KH ₂ PO ₄	170
	CaCl ₂ .2H ₂ O	440
	MgSO ₄ .7H ₂ O	370
	(NH ₄) ₂ .5O ₄	
Micro	FeSO ₄ .7H ₂ O	27.8
	Na ₂ EDTA	37.3
	H ₃ BO ₃	6.2
	MnSO ₄ .4H ₂ O	22.3
	ZnSO ₄ .7H ₂ O	8.6
	KI	0.83
	CuSO ₄ .5H ₂ O	0.025
	CoCl _{1.6} H ₂ O	0.025
	Na ₂ MoO ₄ .2H ₂ O	0.25
Vitamins and others	Thyamine- HCl	0.1
	Pyridomin -HCl	0.5
	Nicotinic acid	0.5
	Inositol	100
	Biotin	
	Glycine	2.0
	Casein Hydrolysate	
Sucrose		30 g l ⁻¹
Agar		6-8 g l ⁻¹
pH		5.8



Appendix- II

Analysis of Variance (ANOVA) for Frequency of meristem producing callus, average callus size, frequency of meristems producing shoots, no. of shoots, average shoot length and frequency of meristems producing roots for table 4, 5 and 6.

	Source	Sum of squares	df	Mean square	F-value	Probability
Frequency of meristem producing callus	Factor A	4200.000	2	2100.000	1.3155	0.2700
	Factor B	216833.333	9	24092.593	15.0928	0.000
	AB	30466.667	18	1692.593	1.0603	0.3930
	Error	431000.000	270	1596.296		
	Total	682500.000	299			
Average callus size	Factor A	3.424	2	1.712	0.3806	
	Factor B	313.241	9	34.805	7.7378	0.000
	AB	55.3674	18	3.0746	0.6838	
	Error	1214.456	270	4.498		
	Total	1586.488	299			
Frequency of meristems producing shoots	Factor A	866.667	2	433.333	2.6000	0.0761
	Factor B	833.333	9	92.593	0.5566	
	AB	2466.667	18	137.037	0.8222	
	Error	45000.000	270	166.667		
	Total	49166.667	299			
No. of shoots	Factor A	0.380	2	0.190	2.4783	0.0858
	Factor B	0.497	9	0.055	0.7198	
	AB	1.153	18	0.064	0.8357	
	Error	20.700	270	0.077		
	Total	22.730	299			
Average shoot length	Factor A	141.291	2	70.645	2.0955	0.1250
	Factor B	2574.699	9	28.633	0.8493	
	AB	532.974	18	29.610	0.8783	
	Error	9102.581	270	33.713		
	Total		299			

Frequency of meristems producing roots	Factor A	11466.667	2	5733.333	7.5882	0.0006
	Factor B	46800.000	9	5200.000	6.8824	0.0000
	AB	39200.000	18	2177.778	2.8824	0.0001
	Error	204000.000	270	755.556		
	Total	301466.667	299			

Appendix- III

In case of table 7, Analysis of Variance (ANOVA) for survivability, shoot length and root length.

		Sum of squares	df	Mean square	F-value	Probability
Survivability	Between groups	0.000	4	0.000	999.990	0.000
	Within groups	0.000	15	0.000		
	Total	0.000	19			
Shoot length	Between groups	10562.456	4	2640.614	2.189	0.1197
	Within groups	18097.020	15	1206.468		
	Total	28659.476	19			
Root length	Between groups	603.157	4	150.789	0.918	.000
	Within groups	2463.450	15	164.230		
	Total	3066.607	19			

Appendix- IV

Analysis of Variance (ANOVA) for survivability, shoot length, no. of branch plant⁻¹ and root length for table 8.

		Sum of squares	df	Mean square	F-value	Probability
Survivability	Between groups	6800.00	4	1700.000	2.550	0.0520
	Within groups	30000.000	45	6660667		
	Total	36800.000	49			
Shoot length	Between groups	5304.788	4	1326.197	3.710	0.0108
	Within groups	16087.056	45	357.490		
	Total	21391.844	49			
No. of branch	Between groups	4.120	4	1.030	2.649	0.0454
	Within groups	17.500	45	0.389		
	Total	21.620	49			
Root length	Between groups	986.388	4	246.597	1.548	0.2045
	Within groups	7166.506	45	159.256		
	Total	8152.894	49			

Appendix-V

In case of table 9, analysis of Variance (ANOVA) for survivability, no. of microtuber plant⁻¹ and average diameter of tuber plant⁻¹

		Sum of squares	df	Mean square	F-value	Probability
Survivability	Between groups	17333.333	5	3466.667	1.440	0.2249
	Within groups	130000.000	54	2407.407		
	Total	147333.333	59			
No. of microtuber	Between groups	2.718	5	0.544	0.526	.000
	Within groups	20.667	20	1.003		
	Total	23.385	25			
Diameter of tuber	Between groups	9.303	5	1.860	0.641	.000
	Within groups	58.036	20	2.902		
	Total	158424.884	25			

Appendix -VI

**For table 10, analysis of Variance (ANOVA) for survivability, shoot length,
no. of tuber, average diameter of tuber, average weight of tuber.**

		Sum of squares	df	Mean square	F-value	Probability
Survivability	Between groups	0.000	7	0.000	999.99	0.000
	Within groups	0.000	26	0.000		
	Total	0.000	33			
Shoot length	Between groups	47841.442	7	6834.492	10.72	.000
	Within groups	16576.531	26	637.559		
	Total	64417.973	33			
No. of tuber	Between groups	199.950	7	28.564	7.242	.0001
	Within groups	102.550	26	3.944		
	Total	302.500	33			
Diameter of tuber	Between groups	48.695	7	6.956	0.906	.000
	Within groups	199.686	26	7.680		
	Total	248.381	33			
Weight of tuber	Between groups	94.201	7	43.457	13.096	.000
	Within groups	26.717	26	1.028		
	Total	120.918	33			

FIGURES



Fig. 4. Meristem isolation from potato sprouts



Fig. 5. Shoot and root formation from isolated meristem at $6 \text{ mg l}^{-1} \text{ GA}_3$ + $0.5 \text{ mg l}^{-1} \text{ BAP}$ + $0.5 \text{ mg l}^{-1} \text{ NAA}$



Fig. 6. Plantlets formed *in vitro* from the variety Smart at $5 \text{ mg l}^{-1} \text{ GA}_3$ + $0.1 \text{ mg l}^{-1} \text{ NAA}$



Fig. 7. Plantlets formed *in vitro* from apical buds.



Fig. 8. Microtuber formation at
8% sucrose + 5.0 mg l⁻¹
BAP + 500 mg l⁻¹ CCC.



Fig. 9. Microtuber formation at
7% sucrose + 5.0 mg l⁻¹
BAP + 500 mg l⁻¹ CCC.



Fig.10. Microtuber formation at
10% sucrose + 5.0 mg l⁻¹
BAP + 500 mg l⁻¹ CCC

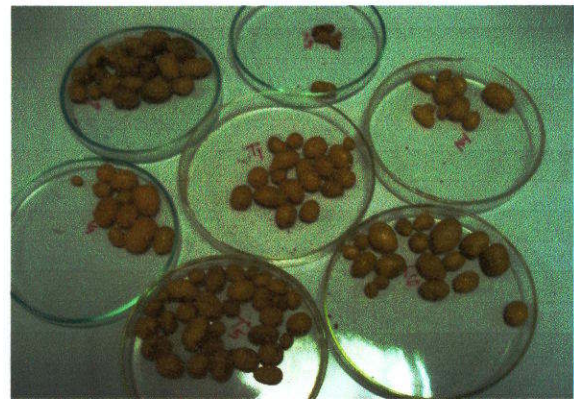


Fig.11. Harvested Microtubers from *in vitro* grown plantlets.

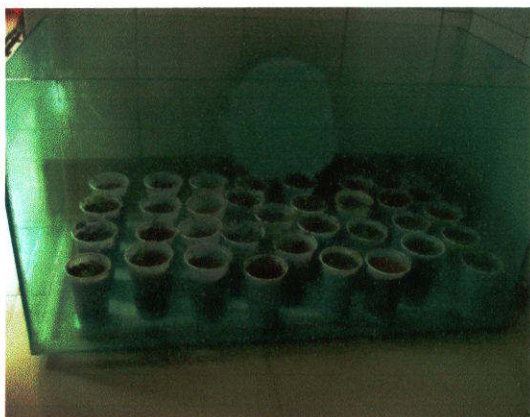


Fig. 12. Transferred plants into plastic pots from *in vitro* grown plantlets were kept into glass jar for 24 hours.



Fig. 13. Transferred plants into green house after 24 hours.



Fig. 14. Plants grown on field directly.



Fig. 15. Harvested minitubers.

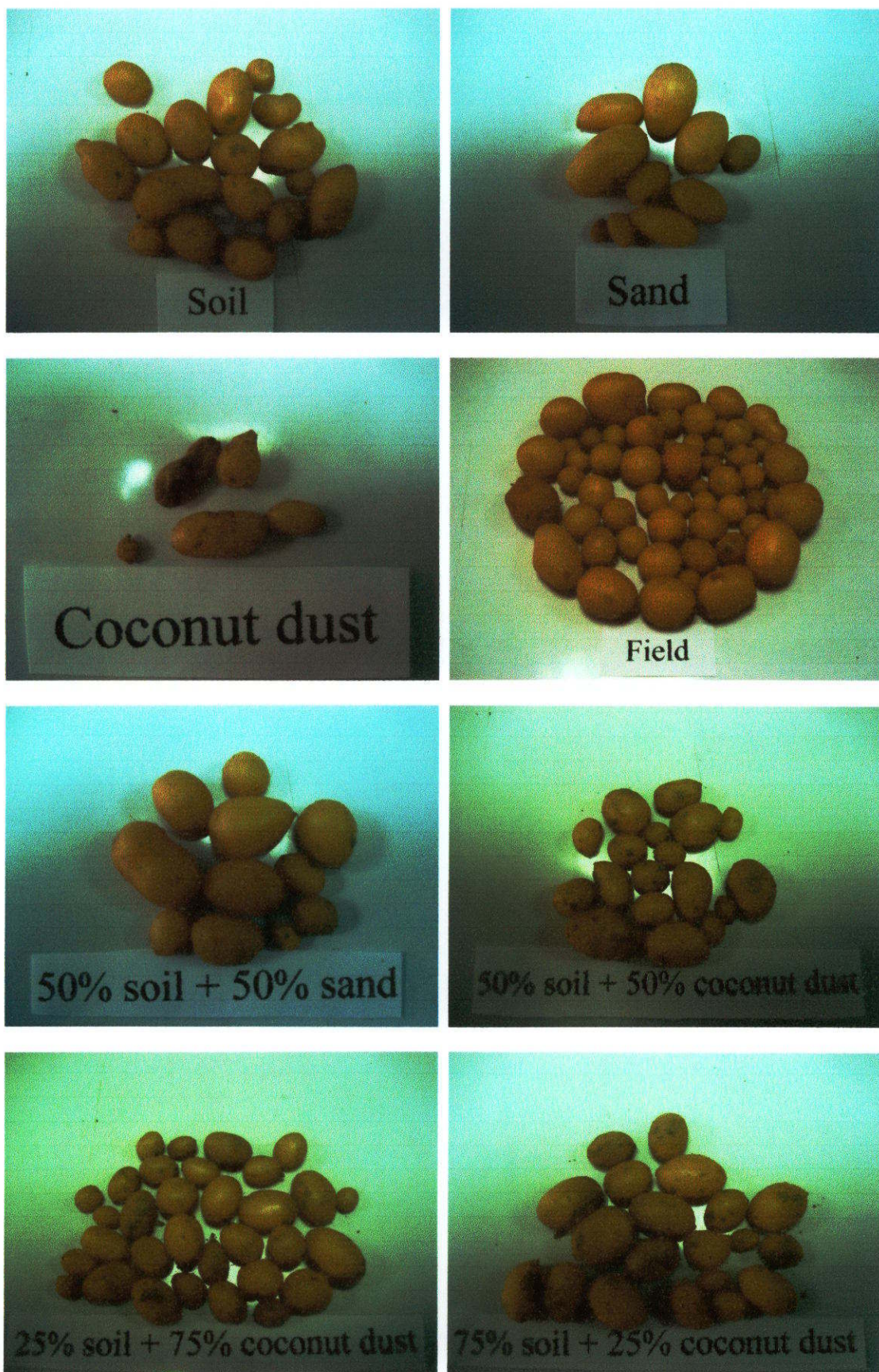


Fig. 16. Minituber formation from different substrates in *ex vitro* condition.

Bangladesh

Potato Production by Area and Average Yields
of Long-Established and Recently Introduced Varieties

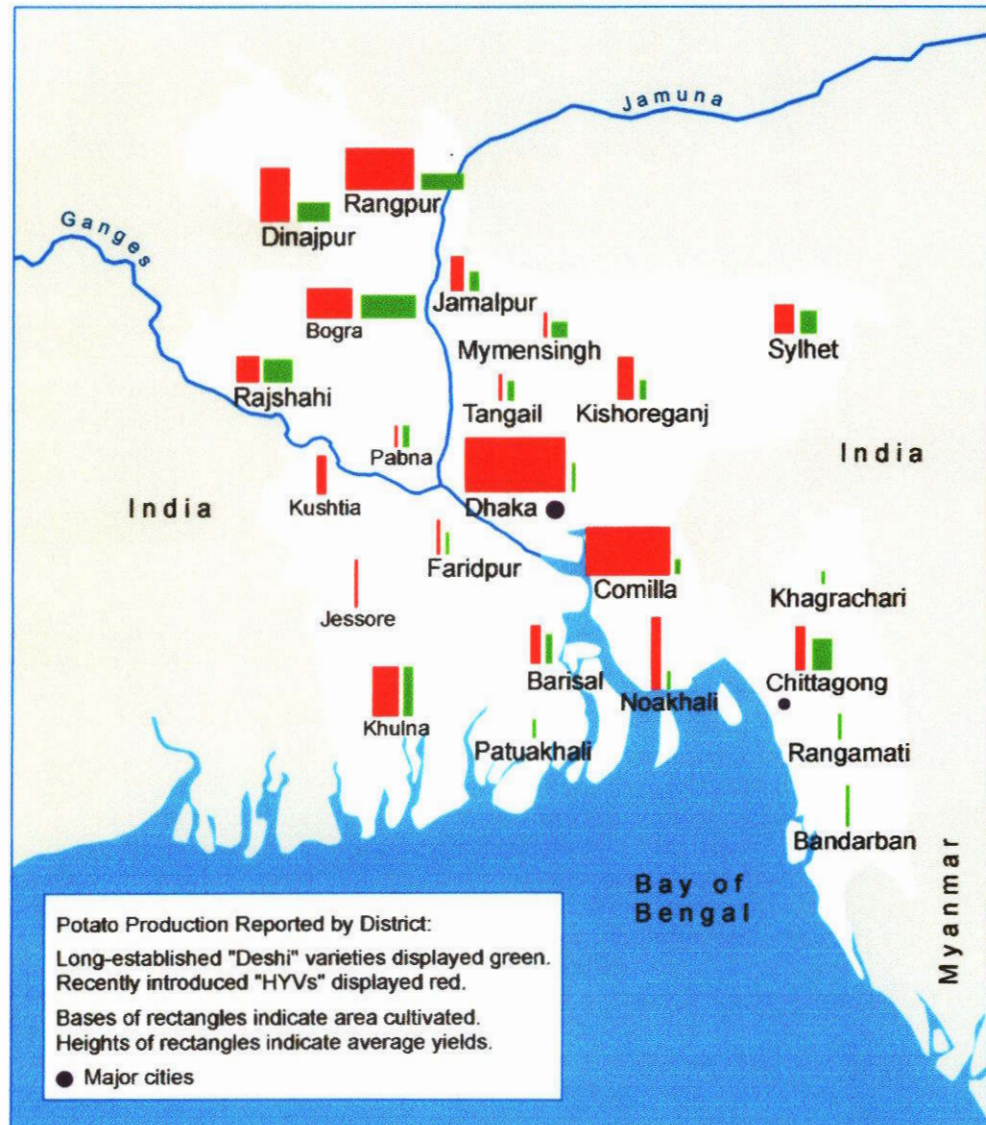


Fig. 17. Potato producing areas in the Bangladesh.