MERISTEM CULTURE AND *IN VITRO* PLANT REGENERATION OF SOME POTATO (*Solanum tuberosum* L.) GENOTYPES

BY

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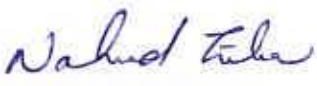

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CERTIFICATE

This is to certify that thesis entitled, **"MERISTEM CULTURE AND *IN VITRO* PLANT REGENERATION OF SOME POTATO (*Solanum tuberosum* L.) GENOTYPES"** *submitted to the faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* **MASTER OF SCIENCE** in **GENETICS AND PLANT BREEDING**, *embodies the result of a piece of bona fide research work carried out by Miss* **SHAMMANA AHMED**, *Registration No.05-1835 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

.....
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Dated: December, 2010
Place: Dhaka, Bangladesh

Dedicated

To my

Beloved Parents

LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation	Full words
^{0} C	Degree Celsius
%	Percentage
1N	1 Normal
µM	Micro mole
2, 4-D	2, 4- dichlorophenoxy acetic acid
Agric.	Agriculture
Agril.	Agricultural
BAP	6- benzyle aminopurine
BBS	Bangladesh Bureau of Statistics
Cm	Centimeter
Contd.	Continued
CRD	Completely Randomized Design
cv.	Cultivar
CIP	International Potato Center
Conc.	Concentration
DAI	Days after inoculation
Dw	Distilled Water
DMRT	Duncan Multiple Range Test
e.g.	Exempli gratia (by way of example)
et al.	et alu (And others)
etc.	et cetera (means and the rest)
FAO	Food and Agricultural Organization
Fig.	Figure
G	Gram
g/l	Gram per litre
Ha	Hectare
ha^{-1}	Per Hectare
h.	Hours

LIST OF ABBREVIATIONS AND SYMBOLS (Cont'd)

Abbreviation	Full words
$HgCl_2$	Mercuric chloride
i.e.	ed est (means that is)
KIN	Kinetin
IAA	Indole-3-Acetic Acid
NAA	α-napthalene acetic acid
NaCl	Sodium Chloride
Int.	International
J.	Journal
Mg	Milligram (s)
mg/l	Milligram per liter
Ml	Milliliter
MS	Murashige and Skoog
GA3	Gibbreline Acetic Acid 3
NaOH	Sodium Hydroxide
No.	Number
NS	Not Significant
pH	Negative logarithm of hydrogen ion concentration (-log $[H^+]$)
PGRs	Plant Growth Regulators
SAU	Sher-e-Bangla Agricultural University
Sci.	Science
Univ.	University
Viz.	Namely
W/v	weight/volume
ZR	Zeatin Riboside

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Meristem culture and *in vitro* plant regeneration of some potato (*Solanum tuberosum* L.) genotypes

ABSTRACT

The experiment was undertaken with a view to standardize a protocol for *in vitro* culture and plant regeneration using potato meristem as explants. The experimental materials were six potato genotypes viz, Shil Bilati, Kufri Shinduri, Lal Pakri, Shil Pakri, Jam Alu and Pahari Pakri. Four levels of GA3 (100, 200, 300 and 400 ppm) were used to assess the influence of GA3 on sprouting abilities of six potato genotypes. The maximum sprouting efficiency was observed in 400ppm GA3 treatment. Combinations of GA3, 2, 4-D and Kinetin were used along with fresh MS media to inoculate meristems and to induce callus. Maximum size of callus was observed (0.82 cm) in Kufri Shinduri inoculated in T4 (0.5 mg/l GA3 +1.0 mg/l 2, 4-D+ 1.5 mg/l KIN). Shil Pakri meristem inoculated in hormonal treatment T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN) showed best results regarding highest single shoot length/plantlet (5.88 cm). Three levels of GA3 treatments (fresh MS, MS+0.5mg/l GA3 and MS+1.5mg/l) were used to study subsequent shoot elongation and plant regeneration of single shoot/plantlet produced from meristem culture. Treatment T2 (0.5 mg/l GA3) gave maximum plant height (5.68 cm), number of branches/plantlet (2.60), root length/plantlet (8.74 cm) and number of leaves/plantlet (14.86). Shil Pakri gave maximum performance in respect of plant height (8.96 cm), number of branches/plantlet (2.78), number of roots/plantlet (4.33) and number of leaves/plantlet (22.56). Shil Pakri treated with T2 (0.5 mg/l GA3) showed maximum plant height (9.50 cm), number of branches/plantlet (5) and number of leaves/plantlet (33.67). Among all genotypes Shil Bilati was least responsive whereas Shil Pakri showed better overall performance from meristem culture to establishment of plants in field conditions. Plantlets obtained from meristem culture were tested using DAS-ELISA protocol for confirming plantlets to be virus free. All the potato genotypes except Shil Bilati and Jam Alu were reported virus free.

Chapter 1
Introduction

CHAPTER 1

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a highly heterogeneous, vegetatively propagated herbaceous plant under the family Solanaceae and belongs to the genus Solanum. The cultivated *Solanum tuberosum subsp. tuberosum* is considered to be originated from Andean and Chilean landraces developed by pre-Colombian cultivators. The wild species progenitors of these landraces probably derived from a group of near about 20 similar wild species referred to as *S. brevicaule* complex, distributed from central Peru to northern Argentina (Spooner *et al.*, 2005). Potato is considered to be the fourth major food crop of the world and is consumed as staple food in more than fourty countries of the world. Potato is the only major tuber crop that is grown in temperate regions. It is also most important tuber crop in terms of production, accounting for about 45% of the total world production of all tuber crops (Shewry, 2003).

Potato is an important food and cash crop in Bangladesh. In Bangladesh it ranks third and after rice and wheat with respect to land under cultivation amongst all food crops (Solomon and Barker, 2001); while ranking first both in area and production among the vegetable crops grown. Modern varieties (HYV) are now cultivated but traditional varieties still occupy about 35% of the total potato production area (Ilangantileke *et al.*, 2001). In Bangladesh, potato represents about 53% of the total edible vegetables although yield is still lower than potential level as grown in advanced countries (FAO/CIP, 1995). Potato plants in our country are grown in winter season and grown best in sandy loam, loamy soil and organic matter enriched soil. Tuber production is highest at 20 ^{0} temperature and reduces due to raise in temperature, even stops in high temperature. All the varieties of Bangladesh can be grouped into two broad categories. One is local or indigenous potato varieties and the other is exotic potato varieties. There are several good indigenous potato varieties in our country. These varieties are well known for their long dormancy period compared to other HYV and exotic varieties which are imported from other

countries. Traditional or present local varieties are introduced about a century ago in our country. These are the only varieties grown until 1960 when HYVs were introduced (Siddique, 1991). Although these varieties are low yielding due to virus infestation they are still widely grown due to their popularity among growers as because they have longer self life, good keeping quality in farm potato storage, low cost of production, reasonably good yields with low inputs, tolerant to stress conditions like drought, heat etc, and gives yield even when infected with diseases and pests, high market demand and prices and above those of Diamant and Cardinal due to better taste (Ilangantileke *et al.*, 1999-2000).

Potato varieties are susceptible to viral diseases which may cause up to 75% yield loss. Some viruses can decrease the yield by 40% singly and in combination with other viruses, the loss is up to 90% (Salazar, 1996). Potato plants are frequently attacked by viruses such as PVX, PVY, PVA, PVS, PVM and PLRV being the most common though many other viruses can also be found (Khalid *et al.*, 2000). The main causes of yield reduction in Bangladesh are use of disease and virus infected tubers, lack of quality seeds available to farmers, management system, adverse climatic conditions etc. Potato yield can be increased 20-30 % by using virus and disease free quality seeds (Karim *et al.*, 2011).These viral diseases are tuber born. Virus contamination symptoms are difficult to identify on tubers, but are more obvious on the aerial part of the plant (stem and foliage). It is not possible to sort out clean tubers from contaminated ones only on their external aspect. Viruses circulate in the sap, and multiply in the foliage of the plants. Therefore quality tuber seeds have to be produced from plants originating from clean plant materials .Clean plant materials can only be obtained by introducing tissue culture by culturing virus free meristem of plant parts. Meristem culture is very important method to produce virus free plants. The virus-free clone produced more vigorous haulm and about 10% higher yields, attributed to more tubers rather than large ones (Karim *et. al.*, 2011).

Large scale virus free *in vitro* plantlet production through meristem culture is being practiced successfully in many countries of the world and it has become

a powerful tool to produce disease, pest and virus free plants. The *in vitro* technology combined with traditional practices has enhanced the commercial production of virus free seeds which is an important pre-requisite to maximize yield in potato (Bajaj and Sopory, 1986). As there are lacks of virus free quality potato seed supply in the region to meet the demand many Asian countries including Bangladesh are importing a large quantity of foundation and certified virus free potato seed from abroad (Nagib *et al.*, 2003).Using meristem culture methods virus free potato seeds can be produced in our country which can reduce huge amount of money expenditure in import process. Some initiatives have already been taken to produce virus free plantlet production with success. So, there is an urgent need to establish some cultivar wise *in vitro* regeneration protocols using different hormones in laboratories. Experiment conducted to see the performance of meristem culture of indigenous potato varieties under laboratory condition is rare. So it is important to do meristem culture of indigenous potato varieties to get virus free plant as well as production of tuber to increase their yield in field condition which is important to make these varieties more popular. Considering all the situations stated above present experiment was conducted with the following objectives:

- To study different hormonal treatments for meristem culture, callus induction.
- To establish an *in vitro* regeneration protocol of some indigenous potato genotypes.
- To find out best hormonal treatment for meristem culture.
- To study rapid multiplication of virus free potatoes relatively in short period of time through micro-propagation.

Chapter 2

Review Of Literature

CHAPTER 2

REVIEW OF LITERATURE

The potato (*Solanum tuberosum* L.) as a vegetatively propagated crop is prone to cumulative infection by bacteria, fungi and viroids a process commonly referred to degeneration. Virus diseases have been recognized as a limiting factor in potato production worldwide. The successful production of potatoes for nutrition and seed purposes demands the control of these viruses which cannot be sufficiently attained by any physical or chemical agent. Tissue culture more specifically meristem culture is an important technique of biotechnology and has a potential to improve the quality and quantity of vegetative propagated potato plants. Biotechnology approaches are now practiced worldwide. Therefore, the literatures, which are most relevant and available to present study, have been reviewed here under following heads.

2.1. Concept of Plant tissue culture:

Tissue culture and plant regeneration are an integral part of most plant transformation strategies which is now widely used for improvement of different crops. Practically any plant transformation experiment relies on the ability to regenerate plants from isolated cells or tissues *in vitro* or using tissue culture techniques (Barcelo *et al.*, 2001).

Tissue culture is a process by which small fragments of live tissue, called explants, are cultivated under aseptic conditions in a culture medium. Suitable recipients are kept in environments under controlled luminosity and temperature. This technique is available to breeders and can be used in practically all stages of a breeding program, from preservation and interchange of genetic resources and increase of the genetic variability to the selection and multiplication of superior genotypes (Cirino and Riede, 1999).

The systematization of genetic breeding programs started in the beginning of the Century with the rediscovery of the basic principles of mendelian segregation which set out the basic laws of genetic heredity. Since then, the application of genetic principles to the development of plants with superior agricultural performance, through the application of most diverse methods, has been systematic. At the same time, there has been considerable progress in *in vitro* plant cell and tissue culture techniques (Binsfeld, 1999).

Experiments with tissue culture began in the nineteenth century when two German biologists, M. J. Shleiden and T. Shwann, reported that the whole plant can be reconstituted whenever cells from some plants were removed (Bonga and Aderkas, 1992).

This experiment led to the concept of totipotency, suggesting that each cell is a unit capable of originating a new organism, and that each cell from a multicellular organism retains the information present in the fertilized ovule. Totipotency stimulates the regeneration of plants with small tissue mass and isolated cells and consequently, undetermined plant cells may show totipotency, plus a high degree of plasticity to physical and environmental stimulus (Cirino and Riede, 1999).

The year of 1934 was the turning point for plant tissue culture principles, especially for the potential unlimited and undifferentiated growth principle. White (1943) cultivated tomato roots in a defined nutritive medium and Gautheret (1934) planted three species of callus (dedifferentiated) from the cambium regions (Cirino and Riede, 1999).

2.2. Plant tissue culture and plant breeding:

Plant breeding aims at developing new cultivars adapted to the stable and high yield cultivation conditions required by high quality production. From the methodological point of view, plant breeding is applied genetics and has been considered the art and science of altering plants genetically for human consumption (Binsfeld, 1999; Barros, 1999).

Despite the extraordinary contribution of the conventional methods to plant breeding, there has been a consensus that significant gains cannot be expected from selection by these processes only (Barros, 1999).

It is stated that, the application of biotechnology techniques allied to conventional plant breeding methods can contribute to the sustainability of agriculture by producing cultivars which are more compatible with the environment. This contribution is especially important to developing countries where the technological resources required to deal with problems related to tropical crops are scarce (Barros, 1999).

Classical methods developed new cultivars until the mid 1980s. Since then, plant breeding has developed several techniques based on plant tissue culture and molecular biology. These techniques became important tools to help breeders look for the allelic diversity needed in a breeding program, enabling them to transcend the primary genetic pool used in classic plant breeding and to incorporate the new alleles into the genome to determine the required characteristics of the culture (Binsfeld, 1999).

Currently, sexual crossing is necessary among wild and cultivated species which are normally incompatible. In these cases, crosses are not possible, and the genetic flow is impeded. However, certain *in vitro* techniques can solve these problems, quickly regenerating many types of useful plants which would otherwise be obtained after many years of intensive breeding cycles (Rogo and Faria, 2001).

2.3. Concept of meristem culture:

The apical meristem together with one to three young leaf primordia, measuring 0.1-0.5mm, has been referred as meristem-tip. The distribution of viruses in plants is uneven. In infected plants the apical meristems are generally either free or carry a very low concentration of viruses. Meristem-tip culture although mainly used for virus elimination, it has also enabled plants to

be freed from other pathogen, including mycoplasmas, bacteria and fungi. Although the apical meristems often free of viruses, there are evidences that suggest some viruses actually invade the meristematic region of the growing tips (Mellor and Smith, 1987).

It was found by Espinoza *et al.*, (1992) that, the meristem in the virus infected plants have a minimum concentration of virus or are virus free and the best control method for potato viruses is production of healthy plants from meristem culture.

The first group of transgenic cultivars commercialized in several parts of the world was resistant to herbicides, insects and pathogens, and were developed by tissue culture combined with molecular biology methods. Thus the researcher had to define the best strategy to solve the problems at hand, always looking for the simplest and the most practical and economically viable alternatives (Ferreira *et al.*, 1998).

In 1943 White first reported about the production of virus free plantlets with tissue culture methods and showed that mosaic virus concentration in the old region of tomato roots is less than the younger region.

Potato virus free clones with the help of meristem culture methods was conducted by Ebadi *et al.* (2007) who cultured meristem derived plantlets in MS medium containing 0.5 mg/l GA3 and 0.4 mg/l NAA and produced regenerated plants with single node culture method.

Hegde *et al.* (1999) conducted an experiment where apical meristems (0.1 to 0.2 mm in length) of seven important Indian potato cultivars, viz. Kufri Bahar, Kufri Chandramukhi, Kufri Chipsona-2, Kufri Giriraj, Kufri Jyoti, Kufri Pukhraj and Kufri Sutlej, were cultured *in vitro* on Murashige & Skoog medium directly without thermo and chemotherapy. The mericlones were indexed employing immunosorbent electron microscopy (ISEM). All these parental stocks were originally free from potato viruses A, M, Y and leaf roll but had infection of PVX and PVS. Success rate was of total virus elimination varied from 0 to

77%, depending on the cultivar and probably also on the virus filter in the parent stock. Mericlones once tested negative to the potato viruses with ISEM continued to remain negative upon subsequent sub culturing over a period of 2 years.

2.4. Effect of meristem size for meristem culture:

Shakya *et al.* (1992) through conducting an experiment eliminated Potato viruses such as Potato virus X (PVX), Potato virus Y (PVY) and Potato virus S (PVS) from the infected tubers of (*Solanum tuberosum* L.) cv. Cardinal by meristem culture. The meristems of various sizes (0.1-1.0 mm diameter) were excised and cultured on the Murashige and Skoog (MS) media containing Benzyl Amino Purine (BAP), Kinetin and Naphthalene Acetic Acid (NAA) in various combinations. Survival of the meristem was influenced by the culture medium, kind and size of the meristem excised. Higher survival percentage (40.5%) was obtained from apical meristems compared to the lateral meristems (25.9%). It was also mentioned that the smaller meristems (0.11-0.25 mm diameter) were more effective than the larger ones in producing virus-free potato plantlets.

In an experiment conducted by Ghai *et al.* (2004) apical meristems (0.2 and 0.5 mm) of commercial potato cultivars Kufri Badshah, Kufri Jyoti and Kufri Chandramukhi were cultured on half-strength MS medium with different supplements of growth hormones (kinetin and GA3). Basal medium did not support survival and growth of small sized (0.2 mm) meristems. Addition of 0.5 mg kinetin/litre alone to the medium was ineffective to increase survival and percent regeneration. MS medium supplemented with 0.2 mg kinetin + 10 mg GA3/litre was most suitable for regeneration. Kufri Badshah was found most responsive to meristem culture followed by Kufri Chandramukhi and Kufri Jyoti.

2.5. Effect of GA3 treatment in potato sprouting:

Jing in 2004 published a paper stating an experiment conducted on Seed tubers of potato cv. Favorita which were subjected to two GA3 treatments to investigate their effects on seed germination rate. Seed tubers treated with GA3 solution (20 mg/l) kept in dark room followed by spraying of GA3 (20mg/l) after 7 days showed 96.75% germination rate. The seed tubers sprayed with GA3 solution (20 mg/l) for 1 hour and kept them in a dark room showed 98.75%. The culture conditions of all treated seed tubers in the dark room were the same.

2.6. Effect of Hormonal combinations and culture environment:

An experiment was conducted to find out the best hormonal combination for meristem culture and *in vitro* regeneration in three cultivars namely Diamant, Cardinal, Multa and Lalpakri (Nagib *et al.*, 2003).Among different types of hormonal combinations 0.5 mg/l GA3 and 0.04 mg/l KIN combination was found to be best medium for the primary establishment of meristem. The primary established meristems were sub cultured on MS_0 medium and MS medium containing BA and IBA singly or combinations. Considering all treatments singly use of IBA (0.5 mg/l) was recommended for proper shoot and root development from primary meristem. In the experiment it was also found that 2.0 mg/l GA3 was the best media for shoot initiation and combinations of GA3 (0.1 mg/l) + KIN (0.1 mg/l) was most effective for high frequency of root formation for studied varieties. Substantial yield increase was also observed in meristem derived plants over their source plants (Nagib *et al.*, 2003).

An experiment was conducted by Sheng *et al.* in 2003. In this experiment stem tip meristems with two leaf primordia of two sweet potato cultivars, Yushu 4 and Yushu 12, were cultured for 20 days on MS + BA (0.5 mg/litre) + IAA (0.1 mg/litre) + GA_3 (0.1 mg/litre). They were then sub cultured on MS medium supplemented with IBA or NAA (0.1, 0.5 and 1.0 mg/litre) at 22, 25, 28, 31 and 34 ^{0} C and a day length of 12, 14 and 16 h. The optimum

temperature and day length were 28^0 C and 14 hrs/day for plantlet regeneration. Supplementation of IBA or NAA to the MS medium at the rate of 0.1-0.5 mg/litre was observed beneficial to plantlet regeneration and the effect of IBA was found better. Supply of IBA at 0.5 mg/litre increased the regeneration rate by 9.9-15.4% as compared with the control and reduced the mean time for plantlet formation by 3.3-8.5 days. A higher concentration (1.0 mg/litre) of the auxins resulted in the formation of numerous calluses on the explants.

Badoni and Chauhan (2009) conducted an experiment on Potato (*Solanum tuberosum*) cultivar Kufri Himalini where meristem tips were cultured on MS medium supplemented with different hormonal combinations i.e. MSGN1 (0.25 mg/l GA3 and 0.01 mg/L NAA), MSGN2 (0.25 mg /l GA3 and 0.03 mg/l NAA), MSGN3 (0.25 mg/l GA3 and 0.04 mg/l NAA), MSKN1 (0.01 mg/l Kinetine and 0.1 mg/l NAA),MSKN2 (0.001 mg/l Kinetine and 0.1 mg/l NAA) and MSKN3 (1mg/l Kinetine and 0.1 mg/l NAA) which effected *in vitro* propagation of potato. It was found that lower concentrations of auxin (0.01 mg/l NAA) with Gibberelic Acid (0.25 mg/l GA3) are best for development of complete plantlets and multiplication from meristem tips.

Zaman *et al.* (2001) observed the effects of three different auxins viz. NAA, IAA and IBA each at four levels (0, 0.1, 0.5 and 1.0 mg/l) was evaluated on meristem culture of potato for production of virus free potato plantlets. Maximum plantlet height (8.3 cm), largest number of nodes/plantlet (7.3) and highest number of leaves/plantlet were recorded at 0.5 mg/l NAA followed by 1 mg/l IBA. Whereas extensive number of roots/plantlet (23.7) as well as the earliest microtuber formation (17 days after transplantation) were recorded at 1.0 mg/l IBA followed by 0.1 mg/l NAA.

In other experiment meristem explants of potato genotypes were *in vitro* cultured on liquid and solid media supplemented with kinetin, abscisic acid, benzyladenine and gibberellins, each at 0, 0.05, 0.1, 0.5, 0.7 and 1 mg/litre. The regenerated shoots were transferred on culture media supplemented with NAA, 2, 4-D, IBA and IAA, each at 0, 0.05, 0.1, 0.5, 0.7 and 1 mg/litre. There

were significant differences among genotypes, hormones, culture media and meristem length. The 0.6 mm meristem of cv. Sante + agar media + 0.7 mg benzyl adenine/litre was the best combination for shoot induction. It was also found that, NAA at 0.7 mg/litre induced the highest shoot regeneration which was 76% (Peyman *et al.* 2004).

The effects of NAA, IAA and IBA at 0.0, 0.05, 0.15, 0.25 and 0.35 mg/litre on the meristem culture of potato (*Solanum tuberosum*) for the production of virus-free plantlets were studied by Ghaffoor *et al.* in 2003. The parameters evaluated were plantlet height, number of node per plantlet, number of leaves per plantlet, root length and number of roots per plantlet. The greatest plantlet height (9 cm) was obtained with NAA at 0.15 mg/litre, whereas the highest number of nodes per plantlet (9.714) was obtained with IBA at 0.35 mg/litre. The number of leaves per plantlet was found greatest (6.143) with IAA at 0.25 mg/litre. The presence of PVX (potato virus X), PVY (potato virus Y) and PLRV (potato leaf roll virus) in 7 of the regenerated plantlets was analyzed by ELISA. Only one plantlet (GH-06) was positive for PVX. The plantlets were not infected by PVY and PLRV.

Orthogonal design with 3 factors at 5 levels was adopted to screen optimal plant hormone combinations that could induce stem segments to differentiate shoots directly at 25 degrees C, 16 h photoperiod and 1500 lx light intensity. The 3 factors were GA3 (gibberellic acid), NAA and BA [benzyl adenine]. Stem segments of virus-free seedlings *in vitro* of potato cv. Super White were used as the explants and the MS medium was used as basic culture medium. Effects of the 3 plant hormones on callus differentiation was ranked as NAA>BA>GA3. The optimum combination was reported 0.25 mg NAA + 1.5 mg BA + 7 mg GA3/litre (Yushi *et al.*, 2004).

In an experiment conducted by Xian (2005) where stem segments with a single node from *in vitro* plants of Youjin were used as explants. These explants were inoculated on MS (Murashige & Skoog) medium supplemented with different combinations of NAA, BAP (6-benzylaminopurine) and GA3 (gibberellic acid) and cultured for 20 days under a photoperiod of 12-16 h, a

light intensity of 2000-3000 lx and a culture temperature of 20-25^0 C. The plantlet height, leaves/plantlet, stem diameter and roots/plantlet were investigated at 7-day intervals from the inoculation. The best medium formula was the MS medium supplemented with 0.5 mg GA3 and 0.1 mg NAA/litre. Plantlet height, leaves/plantlet, stem diameter and roots/plantlet in this medium were 5.0 cm, 4.2 leaves, 0.2 mm and 2.7 roots higher than those in the MS medium without plant growth regulator (as control) was reported.

2.7. Callus induction:

ZhiJun *et al.* (2005) investigated the effect of auxin, GA (gibberellic acid) and BAP (benzyl adenine) on potato shoot growth under *in vitro* condition. The shoot length of potato explants increased with the increasing of concentrations (0.5-10 mg/dm^3) of IAA treatment especially with the addition of GA3 (0.5 mg/dm^3), but was inhibited by BAP (5 mg/dm^3). The root number and root fresh weight of potato explants increased with the increasing of IAA levels either in the presence of GA3 (treatment IAA + GA) or not (IAA alone). However, no root was observed in the treatment IAA+BAP, instead there were brown swollen calluses formed around the basal cut surface of the explants. The addition of GA3 remarkably increased the fresh weight and diameter of calluses.

Yushi *et al.* (2004) investigated an experiment in china to screen optimum plant hormone combinations that could induce stem segments to differentiate shoots directly at 25^0 C, 16 h photoperiod and 1500 lx light intensity. Stem segments of virus-free seedlings *in vitro* of potato cv. Super White were used as the explants and the MS medium was used as basic culture medium with three different hormones i.e. GA3, NAA and BA. Effects of the 3 plant hormones on callus differentiation was ranked as NAA>BA>GA3. The optimum combination was found to be 0.25 mg NAA + 1.5 mg BA + 7 mg GA3/l.

Omidi and Shahpiri (2003) conducted an experiment to study and determine the effects of growth regulators (2, 4-D and kinetin), cultivar, explant and light

on callus induction in potato. In addition, the effects of cultivar and explant were evaluated on callus organogenesis. Callus induction on internode explants was tested using MS medium supplemented with combination of 1, 2 or 3 mg 2, 4-D/litre and 0.00, 0.01 or 0.10 mg kinetin/litre. Analysis of variance revealed a significant effect of 2, 4-D and kinetin concentration, and their interaction, on the frequency of callus induction and number of roots on the callus. The effects of kinetin concentration and kinetin x 2, 4-D interaction were significant on the initiation time of callus induction and volume of callus. However, the effect of 2, 4-D concentration alone on these variables was found insignificant. The effects of cultivar and explant on callus induction in leaf and internode explants of potato cultivars (Agria, Cosmos, Sante, Concord, Ajax and Diamant) were studied. Leaf and internode explants were planted on MS medium, supplemented with 5 mg 2, 4-D and 0.25 mg kinetin/litre. Analysis of variance revealed significant effect of cultivar and cultivar x explant interaction on callus volume, while the effect of explant was not significant. The effect of cultivar, explant and their interaction on frequency of callus induction was not significant, while the effects of these factors on the initiation time of callus induction was significant. Finally, the effect of light on callus induction in leaf and internode explants was investigated. Callus was mostly induced in leaf explants under dark conditions, but was induced in internode explants under both dark and light conditions. Also in the regeneration stage, the effects of cultivar and explant on callus organogenesis were reported significant.

2.8. Shoot development from meristem culture:

The shoot apical meristem is responsible for primary shoot growth, whereas lateral branching is initiated by the development of axillary meristems. Produced in the axils of leaves, axillary meristems arise post embryonically and are derived either directly from the meristematic cells of the shoot apical meristem of potato (*Solanum tuberosum*) (Sussex, 1955).

2.9. *In vitro* plant regeneration and multiplication:

The numerous and different tissue culture methods were done in potato. These methods allow rapid multiplication of potato clones (Ahloowalia, 1994).

Molla *et al.* (2008) conducted an experiment on direct plant regeneration of potato (*Solanum tuberosum* L.) were ready MS basal medium with vitamins (Duchefa, Netherlands) supplemented with 0.01mg/l IAA, 0.20 mg/l GA3, 44.0 g/l $CaCl_2$ and five different concentration of Zeatin Riboside (ZR) viz, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l. Internodes and were sprouted from proximal and distal ends within 18-21 and 21-28 days respectively without callus formation in 3.0-5.0 mg/l of ZR and 2.0-5.0 mg/l ZR. The maximum 38 ± 5.3 and 8.0 ± 1.7 shoots were recorded from each internode and leaf explant respectively.

It was found that producing mini-tubers from *in vitro* plantlets allows a faster multiplication rate in seed tuber production programs and reduces the number of required field generations (Imma and Mingocastel, 2006).

Balali *et al.* (2008) studied the mini-tuber production in the plantlets that originated from virus free sprouts and a genotype of the same cultivar (Marfona) originated from apical meristem. The results showed that the number of mini-tubers per plant was higher for genotypes originated from virus free sprouts.

Sanavy and Moeini (2003) studied the effects of cultivars, NAA, BAP growth regulators in production of minitubers that were obtained through meristem culture. In the study to multiply plantlets which were obtained in meristem culture technique, they cultured single node in the MS solid media containing NAA and BAP. They showed that the best media for single node culture is MS media without any growth regulators.

Bostan and Demirel (2004) conducted experiments to obtain PVX, PVY and PLRV free potato tubers through meristem culture and indicated that, best

medium for potato single node culture after meristem culture is MS medium without any growth regulators.

Pereira, *et al.* (2005) evaluated in experiments the position, presence or absence of leaves and bud numbers of the explants *in vitro* multiplication of potatoes cv. Baronesa. The culture medium was constituted by salts and vitamins of MS, supplemented with 100 mg/l myo-inositol, 30 g/l sucrose and 6 g/l agar. Different types of nodal segments were used (basal and apical, with and without leaves, having one, two and three axillary buds). The material was maintained in a growth room at 25 ± 2 ° C, 16-h photoperiod. For height and number of regenerated sprouts, the best results were obtained with explants originating from the basal position and with three axillary buds. The multiplication rate was higher in explants with a single bud, regardless of the position. Only in basal explants did the presence of leaves improve the multiplication rate. It is concluded that when heterogeneous explants are used, the initial characteristics of the explants can promote variation and cause mistakes in the development of the *in vitro* material.

Petioles, internodes and leaf explants in combination with different plant growth regulators, especially different concentrations of zeatin riboside (ZR), were tested in an experiment conducted by Zel and Medved (1999). It was found that the shoot regeneration was most successful on callus derived from internode tissue cultured on induction medium supplemented with 2.5 mg ZR, 0.2 mg NAA, 0.02 mg gibberellic acid (GA3)/litre for 2 weeks and then transferred to a shoot induction medium containing 2.5 mg ZR/litre. In a comparison of the regenerative potential of Igor with that of Desiree, Igor had poorer and slower regeneration and produced fewer and shorter shoots. However, the protocol established was reported suitable for shoot regeneration for use in Agrobacterium-mediated transformation of Igor.

A one-step regeneration system is described by Rodriguez *et al.*, (2000) using leaf explants of potato cultivars Diacol Capira (DC) and Parda Pastusa (PP). The effect was investigated of different ratios of auxins and cytokinins added to a basal medium (Murashige and Skoog basal salt mixture supplemented

with 30 g/litre sucrose, 0.5 g/litre thiamine, 1 mg/litre gibberellic acid, 40 mg/litre ascorbic acid, and 1.7 g /litre, phytagel, and a pH of 5.7. All leaf explants from DC treated with zeatin riboside (3 mg/litre) and indole 3 acetic acid + IAA (1 mg/litre), and all leaf explants from PP treated with zeatin riboside (3 mg/litre) induced regeneration, producing green and morphologically normal plants.

In an experiment one, two and three-step methods of plant regeneration from stem culture of potato cv. Delaware were tested as methods for producing plant material for gene transformation. Results showed that the one-step procedure using thidiazuron, a synthetic cytokinin, was the best for rapid plant regeneration. In this culture medium, several buds and shoots were regenerated from stem culture, while the other methods using a culture medium supplemented with combinations of GA, BAP (benzyladenine), NAA, zeatin, 2ip (isopentenyladenine) and IAA produced white and green callus. Morphology and chromosome number of all regenerated plants were similar to the original plants. Results showed that the regeneration system was suitable for cv. Delaware and that the culture conditions prevented genetic variation of the regenerated plants (Ehsanpour and Jones, 2000).

Three potato cultivars, Cardinal, Altamash and Diamont were selected for *in vitro* responses in an experiment. High regeneration and morphogenic potential of different explants i.e., shoot tips, leaf discs, nodes and internodes have been tested for direct regeneration. Basal media was Murashige and Skoog and different hormonal combinations of benzyladenine and IAA were supplemented. Statistical analysis showed that explant source had significant effect on direct regeneration and the nodal explants had maximum regeneration. The number of shoots obtained from node was 17.6 from Cardinal followed by Diamont 14.3 and Altamash 9.0. Shoot apices also resulted in shoot regeneration comparatively better than leaf discs and internodal explants but lesser than from nodes. The most suitable medium was reported MS with 2.0 mg/litre BAP and IAA at 0.5 mg/litre giving maximum regeneration. It was also found that interaction of cultivars with explant and media is highly significant at 1.0% (Hussain *et al.*, 2005).

Chapter 3

Materials And Methods

CHAPTER 3

MATERIALS AND METHODS

The research experiment was carried out at Tissue Culture Laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207. The duration of experiment conducted was from March 2010 to October 2011 to obtain *in vitro* regeneration of meristem cultured plant materials of six potato varieties. Potato varieties which are grown in different locations of Joypur, Joypurhat and Rangpur were collected from Bangladesh Agricultural Development Corporation (BADC). Following experiments were conducted to fulfill the objectives of the present study.

Experiment 1: Effect of different concentrations of GA3 treatment on sprouting abilities of six potato genotypes.

Experiment 2: Effect of 2, 4-D, KIN and GA3 on meristem culture, callus induction and shoot regeneration of six potato genotypes.

Experiment 3: Effect of three levels of GA3 application for rapid multiplication of single shoot derived from callus.

3.1. Primary plant materials:

The primary experimental materials were six potato genotypes listed below,

Shil Bilati
Kufri Shinduri
Lal Pakri / Fata Pakri
Shil Pakri
Jam Alu &
Pahari Pakri

3.2. General experimental methods:

Primary plant materials of six potato varieties were treated with GA3 (Plate 1) to obtain sprouts from where meristem tips were collected. meristem tip of about 0.2-0.5 mm length were used as initial explant for *in vitro* regeneration of callus under different hormonal treatments. Single shoot induced from callus was sub cultured in MS medium without or with different levels of GA3 concentrations. Further subculture in MS_0 medium with hormones suitable for rapid multiplication of plantlets was done.

3.2.1. GA3 Treatment and collection of explants:

Individual potato varieties were treated with GA3 to germinate sprout. Carefully selected potatoes were used to conduct this experiment. Similar sized potato of each variety was taken. Average weight of these potatoes were 76.33 gm, 14.67 gm, 6.67 gm, 6gm, 4gm and 10.67 gm for Shil Pakri, Kufri Shinduri, Lal Pakri, Shil Pakri, Jam Alu and Pahari Pakri respectively. Before GA3 treatment potato varieties were gently washed with liquid detergent (Trix) and washed thoroughly in running tap water without removing potato skin to remove all kinds of dirt. Then all of them were washed with distilled water and dried in normal room temperature to remove extra moisture on potato skin. Different potato varieties of similar size and weight were selected for spraying four concentrations of GA3 treatments (100, 200, 300 and 400 ppm/l). Spraying was done two times a day regularly for 25-30 days as required. Data was collected regularly from 30 day's old treated potatoes. Among four GA3 treatments 400ppm concentration of GA3 per liter of distilled water was selected for spraying as it gave the most effective result in respect of days to initiate sprouting and number and length of sprouts/ potato (Table1).Results are represented in result and discussion section in Table 1. GA3 concentration of 400 ppm/l were sprayed to get sprouts from where tips of about 0.2-0.5 mm were collected from freshly grown sprouts for the purpose of using as explants for culturing meristem (Plate 4).

3.2.2. Media and hormonal applications:

For culturing meristem tips MS (Murashige and Skoog, 1962) media was used as a basal medium and six different hormonal treatments were used for meristem inoculation i.e.,T1 (0.5 mg/l GA3, 0.5 mg/l 2,4-D and 0.5 mg/l Kinetin), T2 (0.5mg/l GA3, 0.5 mg/l 2,4-D and 1.0 mg/l Kinetin), T3 (0.5 mg/l GA3, 0.5 mg/l 2,4-D and 1.5 mg/l Kinetin),T4 (0.5 mg/l GA3, 1.0 mg/l 2,4-D and 1.5 mg/l Kinetin), T5 (0.5 mg/l GA3, 1.0 mg/l 2,4-D and 0.5 mg/l Kinetin), T6 (0.5 mg/l GA3 , 1.0 mg/l 2,4-D and 1.0 mg/l Kinetin) and T0 (0 mg/l GA3, 0 mg/l 2,4-D and 0 mg/l Kinetin). After shoot regeneration from callus induced from meristem tips, sub culturing was done in three different levels of GA3 hormonal treatments i.e. MS medium and 0 mg/l GA3, MS media and 0.5 mg/l GA3, MS medium and 1.5 mg/l GA3 treatments. Sub culturing of shoots or callus was also done with the best treatment found from experiment 3 for vigorous and rapid multiplication of plantlet derived from meristem culture.

3.2.3. Preparation of Culture Media:

The significant factor for the success of plant tissue culture is selection of appropriate culture media with precise concentrations of nutrient components and growth regulator. For *in vitro* regeneration of potato MS medium (Murashige and Skoog, 1962) was used as basal medium. The table showing composition of MS medium is presented in Appendix-1.

MS medium was used alone or with different concentrations of hormones according to the requirements. MS media was prepared using stock solutions which was prepared and stored at 4±1° C temperature well before the preparation of MS media.

3.2.3.1. Preparation of stock solutions:

MS medium is prepared by combination of stock solutions with different minerals and hormones required for plant growth development. Each stock solution composed of different types and amount of major salts, minor salts,

iron and organic, growth regulators etc. respectively. All the chemicals used for stock solution is highly purified and labeled as plant tissue culture tested grade. The chemicals are dissolved in double distilled water or highly purified de-ionized water. Each chemical are added according to the list of ingredient presented in Appendix-I.

3.2.3.2. Preparation of stock I solution:

Stock solution of macro nutrients or stock I was prepared with 10 times (10X) of the final strength of the medium in 1000ml of distilled water. Salts are weighted accurately and dissolved completely ten times the weight of the salts required for one litre of medium in 750 ml of distilled water. Each chemical was dissolved completely before adding other chemicals. The final volume was made up to one litre by adding distilled water. The stock solution was filtered through what man no. 1 filter paper to remove all the solid particles. The stock I solution was stored in a glass container, tagged with date of preparation and stored in refrigerator at 4± 1° C for future use.

3.2.3.3. Preparation of stock II solution:

The stock solution of micronutrients was made up to hundred folds (100x) the final strength of the medium in 1000ml of distilled water as described earlier same as stock I. The stock solution was filtered as same manner as described earlier and labeled and stored in a refrigerator at 4± 1° C.

3.2.3.4. Preparation of stock III solution:

The stock III solution is the solution of iron-EDTA which was added freshly and made hundred folds (100X) the final strength of the medium in 1000ml of solution. FeSO4 and Na-EDTA were dissolved in 750 ml of distilled water in a beaker by heating on a heater cum magnetic stirrer. The volume was made up to 1000 ml by further addition of distilled water. The preparation and storage of stock solution was done in amber bottle or a bottle completely

wrapped with aluminium foil. The prepared bottle was stored in refrigerator at 4 ± 1° C temperature.

3.2.3.5. Preparation of stock IV solution:

This is the stock solution of vitamins and amino acids prepared by adding Pyridoxine HCl (Vitamins B_6), Thiamine HCl (Vitamins B_2), Myoinositol (Inositol), Glysine and Nicotinic acid (Vitamins B_3) one by one. Each of the vitamins and amino acids except myoinositol were taken at 100 folds (100X) of their final strength in a measuring cylinder and dissolved in 400 ml distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 4 ± 1° C for later use. But the myoinositol was made separately 100 folds (100X) the final strength of the medium in 1000ml of distilled water. This stock solution was also filtered and stored in a refrigerator 4 ± 1° C.

3.2.4. Stock Solution for growth regulators:

Stock solution I, II, III and IV are required to prepare MS medium. There are other different types of stock solution was prepared such as growth regulators (/Hormones). Auxin and cytokinin are growth regulators for adding into MS medium to support good growth of tissue, root, shoot and organ development according to the experiment requirement. In the present investigation three types of growth regulators were used. They are 2, 4-dichlorophenoxy acetic acid (2, 4-D), Kinetin (KIN) and Gibberellic acid (GA3). Following chart shows the powder forms of growth regulators (solute) and their appropriate solvent.

Growth regulators / solute	Solvents
2,4-D (Auxin)	96% ethanol
KIN (Cytokinin)	1 N NaOH
GA3 (Gibberellin)	Ethanol

*GA3= Gibberellic acid, **2, 4-D**=2, 4-dichlorophenoxyacetic acid, KIN= Kinetin.

Above mentioned growth regulators comes in containers in powder forms. To prepare growth regulator stock solution 50mg of powder of each growth regulator were separately dissolved in their respective 50 ml solvents and stored at 4 ± 1° C. Newly prepared growth regulators could be used maximum for two months. After the expired date new growth hormones were prepared for experimental use.

3.2.5. Preparation of other stock solutions:

3.2.5.1. 1 N NaOH preparation:

40 gm of NaOH pellets were dissolved in one litre of Distilled water to prepare 1N NaOH. Prepared solution was stored in cool and dry place in a glass bottle. This solution was used for adjusting pH of final MS media preparation.

3.2.5.2. 70% Ethanol preparation:

In a 100 ml measuring cylinder 70 ml 99.9% ethanol was poured. Double distilled water was poured up to the level of 100 ml. This solution was made fresh each time before use.

3.2.6. Preparation of MS media:

One litre of MS medium was prepared according following steps stated below:

- 100ml of stock I, 10ml of stock II, 10ml of stock III and 10ml of stock IV were poured one by one in a one litre beaker which was put on a hot plate magnetic stirrer.
- 500ml of double distilled water was added into the beaker.
- 30 gm sucrose added to the beaker and with a help of magnetic stirrer sucrose was completely dissolved slowly.
- Different concentrations of hormonal concentrations were added according to the experimental requirements.

- The prepared mixture was poured in one litre of measuring cylinder and the volume was made up to the level by adding double distilled water.
- Then the pH of the mixture was adjusted to 5.8 using a pH meter. For adjustment of the pH 1 N NaOH and 0.1 N HCl were used as per requirement.
- 8 gm Agar was added to the mixture and heated in a micro wave oven at 100 ° C slowly until the agar dissolves completely.
- The MS medium is now ready to sterilize and after sterilization it is ready to use.

For sterilization the hot MS medium is dispensed into culture vessels (of about 20ml media/ Vessel) or test tubes (of about 10ml media/test tube). After distribution of media the test tube was wrapped with aluminium foil and each test tube was marked for hormonal combinations with a help of glass marker pen.

3.3. Sterilization steps:

3.3.1. Sterilization of culture media:

The culture vessels or test tubes with newly prepared culture media were sterilized at 15 psi pressure at 121 ° C for 20 minutes in an autoclave machine. The medium was cooled at room temperature before use.

3.3.2. Sterilization of glassware and instruments:

All types of glassware, measuring cylinder, test tube, culture vessels etc and all metal instruments were washed with liquid detergent and cleaned with running tap water and then washed again with distilled water. All glassware's were autoclaved at 15 psi pressure at 121 ° C for 30 minutes. All the metal instruments which is to be used in laminar air flow during culturing and outside laminar air flow during doing various works in media preparation, chemical

measurement etc after washing were wrapped with aluminium foil and autoclaved at 15 psi pressure at 121 ° C for 30 minutes.

3.3.3. Sterilization of culture room and transfer areas:

Before using culture room it was carefully cleaned with detergent and wiped with 70% ethanol. The room was sprayed with formalin. Great precautions was taken during the spraying of formalin such as, wearing protective mask, covering the whole body with protective cloths, wearing gloves , protective glasses etc to protect the skin from any harm cause by this chemical. After spraying the whole room it was concealed for 24 hours. Then after entering the room all the rakes and shelves were surface cleaned with 70% ethanol. This procedure was conducted at regular intervals.

3.3.4. Sterilization of laminar air flow cabinet:

The laminar air flow cabinet was started half an hour before working and sterilized well beforehand. The Air flow cabinet surface was cleaned with cotton soaked with 70% ethanol. The lead of cabinet then closed well and UV was switched on while turning off the air flow. The UV light of cabinet was left on for 30 minutes. After the 30 minutes the surface area is wiped clean with 70% ethanol. For reducing the incidence of contamination aluminium foil wrapped pre-sterilized all equipments which was used during culturing kept in airflow cabinet switching on the UV light for another 10-15 minutes just before starting culture. During the culture all the equipments were frequently flame sterilized after dipping into 70% freshly made ethanol. Hands were washed properly and sterilized with 70% ethanol

3.4. Environment of culture room:

For keeping Meristem culture and sub cultured Vessels and test tubes a controlled environment were organized. The temperature of culture room was maintained within 25 ± 1 ° C with the help of air conditioner. Photoperiod of 16

hours was maintained with the help of white florescent lights and 100W bulbs. The light intensity was maintained 3000 lux and monitored using lux meter.

3.5. Culture methods:

The general meristem culture procedure was carried out as follows:

3.5.1. Explants:

For collection of explants, potato tubers treated with GA3 (400ppm/l) was left for sprouting. When a sprout attains 0.5 – 1cm in length, meristem was collected from sprouts for culturing. Meristem was the starting material for culture.

3.5.2. Surface sterilization and meristem culture:

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Potato sprouts were excised with sterilized surgical blade and collected in to six different 50ml beaker to take six varieties which was marked well with marker pen. Sprouts were washed with distilled water two times. Then washed sprouts were taken under laminar air flow which was been sterilized before starting culture. For surface sterilization sprouts were again washed with sterilized double distilled water. Then was sprouts were kept in 70% ethanol for 1 minute then washed three times with double distilled water. Then these sprouts were immerged into 0.1% HgCl2 solution in addition to 3 drops of Tween-20 for 4 minutes. After 4 minutes sprouts were washed with double distilled water four times to remove any sort of chemicals and dirt's that exists on sprouts. After carefully removing extra water sprouts were put on clean sterilized Petri dish.

3.5.3. Meristem dissection:

Meristem was taken from shoot tip or sprouts. Shoot tips were covered by numerous soft leaf primordia. Under 50X magnification binocular microscope outer leaf primordia surrounding the meristematic region was discarded using

sharp microsurgical blades and pointed fine needle, until only the meristematic region and one or two leaf primordia about 0.5 mm remains intact (Plate 4). Special care was taken not to damage tissue during dissection of meristem as this part was the most critical and delicate part in the experiment.

3.5.4. Inoculation of meristem:

One by one tip of sprouts were dissected and keeping the cut surface touching the media. Special care was taken to keep the extremely small sized cut pieces of meristem intact and uninjured. After inoculation each test tube was immediately wrapped with aluminium foil. The cultured test tubes were placed in rakes in controlled environment.

3.5.5. Observation and data collection:

Cultured meristem was regularly observed to notice any visible change of growth and any color development. Contaminated or dead cells or unresponsive materials were removed and data was recorded. Each treatment was done in 10 replications and survival rate was recorded. 5 randomly selected replications from each treatment were selected for data collection on regular basis for conducting experiments in next level of plant regeneration experiments. Data was collected on following parameters;

Survival Percentage of explants inoculated (%)
Callus texture and color
Days to callus initiation
30 days old callus size (length and breadth in cm)

After callus induction and regeneration of single shoot on the same culture, data was also taken on regular basis on following parameters;

Days to shoot initiation

Shoot length/callus (14, 21 and 30 DAI)

Days to root initiation

3.5.6. Subculture of single shoot:

Initial sub culturing was done using single shoot (30 DAI) developed from callus in meristem culture to regenerate more shoots where three levels of growth hormones used along with GA3. 21 days after subculture following data was taken on regular basis,

Plant height /plantlet (cm)

Number of branches/ plantlet

Number of roots/ plantlet

Root length/plantlet (Maximum length in cm)

Number of leaves/ plantlet

3.5.7. Subsequent sub culturing

Sub culturing of shoot in media containing hormonal treatment was done which showed promising results and data was recorded on following parameters;

Plant height/plantlet (cm)

Number of branches/ plantlet

Number of roots/ plantlet

Root length/plantlet (Maximum length in cm)

Number of leaves/ plantlet

3.5.8. Continuous sub culture of shoot cuttings:

After taking above mentioned data continuous sub culturing was done to get vigorous plantlets using 2 cm long shoot cutting. After doing 3-4 sub culturing all the plants showed vigorous growth at high light intensity.

3.6. Treatments:

Three experiments were conducted to assess the effect of sprouting abilities of potatos, the effect of different concentration of 2, 4-D, KIN and GA3 on callus induction, shoot regeneration and plantlet establishment.

3.7. List of experiments and Data collection methods:

3.7.1. Experiment 1: Effect of different concentrations of GA3 treatment on sprouting abilities of six potato genotypes

Design: CRD
Replication: 3
Explant: Potato tuber
Number of potato used per treatment: 3
Treatment details: GA3ppm/L
100ppm
200ppm
300 ppm
400 ppm
Parameters: Data was collected under following parameters;

3.7.1.1. Days required to initiate sprouting:

During regular spraying of potato it was carefully observed to see any germination of sprouts. Starting date of spraying was recorded for each treatment and each variety. As soon any visible sign of sprouting was seen that day data was recorded and number of days was counted to find out the number of days required to initiate sprouting.

3.7.1.2. Number of sprout / potato:

Depending on the varietal responses after 25 to 30 days of spaying number of sprouts/ potato was counted and data was recorded.

3.7.1.3. Maximum sprout length (in Centimeters):

Maximum sprout length in centimeters was taken and recorded from 30 days old potatoes treated with different concentrations of GA3.

3.7.1.4. Number of sprouts/ eye:

Number of sprouts/ potato was counted and data was recorded from 25- 30 days old potatoes treated with different concentrations of GA3 depending upon varietal responses in different varieties.

3.7.2. Experiment 2: Effect of 2, 4-D, KIN and GA3 on meristem culture, callus induction and shoot regeneration of six potato genotypes.

Design: CRD
Replication: 5
Explant: Meristem / shoot tip from potato sprouts
Number of explants inoculated per replication: 1
Treatment details:

Hormonal Treatment	GA3 + 2, 4- D + KIN (mg/L)
T0 (control)	0+0+0
T1	0.5+0.5+0.5
T2	0.5+0.5+1.0
T3	0.5+0.5+1.5
T4	0.5+1.0+1.5
T5	0.5+1.0+0.5
T6	0.5+1.0+1.0

*GA3= Gibberellic acid, **2, 4-D=** 2, 4-dichlorophenoxyacetic acid,KIN= Kinetin.

In this experiment, the effect of different concentration and combinations of 2, 4-D, KIN and GA3 were used with MS media for inoculating potato meristems

to find out the best combination for meristem culture. In this experiment six different combinations of 2, 4-D, KIN and GA3 were used and a control treatment with no hormonal combination was also used.

Parameters: Data was recorded time to time under following parameters.

3.7.2.1. Survival rate:

Survival rate of inoculated meristems were recorded. Initially ten replications of each treatment of each variety were done. Number of explants managed to survive was recorded. The percentage of meristem survived was recorded using following formula.

$$\% \text{ Survival rate of explants} = \frac{\text{Number of meristem survived}}{\text{Total number of meristem inoculated}} \times 100$$

3.7.2.2. Days to callus initiation:

Newly cultured materials were observed every day. When any change of meristem or development of callus in cultured explants was observed data were recorded as days to meristem germination or days to callus initiation.

3.7.2.3. Callus color:

30 days old meristem culture was observed. In some cases callus was produced. Color of callus was recorded.

3.7.2.4. Callus texture:

Calluses produced under different treatments of 30 days old cultures were carefully observed for their physical characteristics (friable and non friable) and data was recorded.

3.7.2.5. Callus Size:

Length and breadth of 30 days old callus were measured in centimeter (cm) with the help of a measuring scale. Callus length was measured vertically and breadth was measured horizontally. The formula (Thadavong *et al.*, 2002) used for estimating size of callus is given below:

$$\text{Callus size (cm)} = \frac{\text{Length + breadth}}{2}$$

3.7.2.6. Single shoot/ callus:

Single shoot development occurred following growth of callus from meristem to some extent. These cultures were carefully observed for shoot initiation and regeneration. Data was recorded such as days to shoot initiation in these cases. When single shoot was visible date was recorded to find out days required to initiation shoot.

3.7.2.7. Shoot length/ plantlet:

Shoot length of cultured explants was recorded 14, 21 and 30 DAI.

3.7.2.8. Days to root initiation:

The cultures were carefully observed everyday for root development and when any root development and elongation was seen which was treated as days to root initiation.

3.7.3. Experiment 3: Effect of three levels of GA3 application for rapid multiplication of single shoot derived from callus.

Design: CRD
Replication: 3
Explant: single shoot derived from experiment 2
Number of explant inoculated per replication: 1
Treatment details:

Treatment	Medium / hormones
T1	Only fresh MS media
T2	MS + 0.5 mg/l GA3
T3	MS + 1.5 mg/l GA3

Parameters:
Single shoot obtained from meristem culture was sub cultured to obtain vigorous plantlet. For that purpose experiment 3 was conducted to find out the best concentration of GA3 application.

In this experiment, the effect of different concentrations of GA3 on shoot development and multiplication of six potato genotypes were studied. There are 3 levels of GA3 concentrations (0, 0.5 and 1.5 mg/L) which were used. In experiment 3 data was collected according to the following parameters:

3.7.3.1 Plant height in centimeters:

Plant height or length of shoot was measured in centimeter (cm) from the base to top of the explants by a measuring scale. In case of multiple shoots the length of the tallest plantlet was considered as plant height in centimeter. Data was recorded from 21 days old subcultures and the mean was calculated.

3.7.3.2. Number of branches/ plantlet:

Number of branches produced from main shoot of the explants was counted and data was recorded from 21 days old subcultures and mean was calculated.

3.7.3.3. Number of roots per plantlet:

Number of roots per plantlet was counted from 21 days old sub cultured plantlets and mean was calculated.

3.7.3.4. Root length in centimeters:

Root length was measured in centimeter (cm) from base to the tip of the roots from 21 days old subcultures and average length of the roots was recorded and mean was calculated.

3.7.3.5. Number of leaves/ plantlet:

The number of leaves produced in each plantlet was counted from 21 days old subcultures and mean was calculated.

3.8. ELISA test:

Primary plant materials were initially reported virus infected during conducting experiments (Alam, 2011) in field conditions during that time. Virus infected plants observed in field conditions and photo was taken (Plate 2). After completion of the experiment successfully regenerated plantlet samples of each variety was send to the laboratory of Bangladesh Agricultural Research Institute, Gazipur for ELISA (Enzyme linked Immunosorbent Assay) test using DAS-ELISA protocol (BARI, 2012) to confirm that the plantlets obtained from meristem culture were virus free.

3.9. Acclimatization:

Well developed plantlet with developed root was removed from the culture vessels carefully without damaging the roots. Any form of culture medium was washed away from the roots with running tap water. And the roots were washed with distilled water. These plantlets were transferred to small tray containing 20 plantlets with small pot filled with sterilized soil, sand and fully decomposed cow dung and ashes (1:1:1). This tray was kept in the hardening room for 7 days.

3.10. Transfer of plantlets to soil:

After acclimatization of plantlets they were transferred to net house, where proper care was taken for growth and development of potato plantlet. After 20 days plantlet showed maximum vigorous growth and was transferred to the field in normal field condition.

3.11. Experimental design and statistical Analysis of Data:

All the experiments such as meristem culture, callus initiation, plant regeneration, and subculture were done under controlled laboratory condition, which was believed to be uniform in such condition. As such, the experiment was conducted in the completely randomized design (CRD). The collected data on different parameters were analyzed using a MSTAT-C package computer program. The analysis of variance was performed and means were compared by Least Significant Difference (LSD) test for interpretation of results.

Chapter 4

Results And Discussion

CHAPTER 4

RESULTS AND DISCUSSION

Regeneration of potato plantlets from meristem culture offers unique facilities for development of reproduction protocol with a view to supply virus and disease free planting materials in large quantities for large scale cultivation. So the regeneration of potato plantlets through meristem culture and shoot multiplication can play an important role. *In vitro* regeneration of six potato potato genotypes (plate 1) using different combinations of GA3, 2, 4-D and KIN on meristem culture, shoot multiplication were studied. The result obtained from this experiment have been presented and discussed under following headings.

4.1. Collection of potato sprouts using various GA3 treatments on primary plant materials for meristem culture

Meristem collection is one major task in meristem culture technique. In this experiment meristem was collected from potato sprouts (Plate 3). Current experiment was conducted to get maximum sprouts relatively in short periods of time. Four levels of solutions concentrated with GA3 (100, 200, 300 and 400 ppm) was sprayed to six potato potato genotypes. Results have been presented in Table 1. Various treatments showed significant influence during the experiment over days required to initiate sprouting, number of sprouts/ potato, maximum sprout length, and number of sprouts/ eye. Results are discussed under following heads.

4.1.1 Experiment 1: Effect of different concentrations of GA3 treatment on sprouting abilities of six potato genotypes

4.1.2. Days required to initiate sprouting

Shil Bilati, Kufri Shinduri, Jam Alu, Pahari Pakri, Lal Pakri and Shil Pakri took longer period of time (5, 8, 29, 4, 8 and 8 days respectively to initiate sprouting) to respond when treated with 100 ppm GA3 . Whereas Shil Bilati,

Kufri Shinduri, Jam Alu, Pahari Pakri, Lal Pakri and Shil Pakri responded early when treated with 400 pm GA3 (3, 4, 3, 1, 2 and 2 days required to initiate sprouting respectively).

Among six potato genotypes Jam Alu responded slowly (29 days required to initiate sprouting) in 100 ppm GA3 application but responded quickly to initiate sprouting (only in 3 days) in 400 ppm GA3 application. Whereas Pahari Pakri took shortest possible time (1 day) to initiate sprouting when they were treated with 400 ppm GA3 solutions.

From above experiment it was found out that, Jam Alu as most dormant potato and less responsive to 100, 200, 300 and 400 ppm GA3 treatments (29, 14, 9 and 3 days required to initiate sprouting respectively) compared to other potato genotypes. Whereas Pahari Pakri was found to be less dormant and easily responding genotype to 100, 200, 300 and 400 ppm GA3 treatments (4, 2, 2 and 1 days required to initiate sprouting respectively).

4.1.3. Number of sprout/ potato

A significant increase in number of sprouts/ potato observed with increasing concentration of GA3 application. Shil Bilati, Kufri Shinduri, Jam alu, Pahari Pakri, Lal Pakri and Shil Pakri gave maximum number of sprout per potato (10, 27, 5, 18, 5, 7 respectively) under 400 ppm GA3 application but gave minimum number of sprout per potato (5,13,2,6,1,3 respectively) when the concentration of GA3 was reduced to 100 ppm.

Maximum number of sprouts/ potato that is 27 was recorded in Kufri Shinduri and Shil Pakri under 400 ppm GA3 applications. Minimum Number of sprout/r potato (1) was recorded in Lal Pakri under 100 ppm GA3 applications.

The above observations illustrated that increasing level of GA3 treatment enhances sprouting intensity and abilities quickly and effectively.

4.1.4. Maximum sprout length (in Centimeter)

Maximum length of sprout was recorded in Jam Alu and shil Pakri (3.50 cm) under 400 ppm GA3 application and minimum length of sprout was recorded in Jam Alu (0.20 cm) under 100 ppm GA3 application. Results have been presented in Table 1.

Almost all potato genotypes showed significant results in response to 400 ppm GA3 application in respect of days required to initiate sprouting, number of sprouts/ potato, sprout length and number of sprouts/ eye. Almost all potato genotypes gave maximum sprout length, number of sprouts/ potato in fewer days due to the application of GA3 at the rate of 400 ppm which was desirable in this experiment to get maximum number of sprouts comparatively in short period of time. 400ppm GA3 treatment was selected as best treatment and used to get maximum sprouts for the collection of meristematic shoot tip in shortest possible time.

Table 1: Effect of GA3 treatments on sprouting abilities of six potato genotypes

Potato genotype	GA3 treatment (ppm)	Days required to initiate sprouting	Number of sprouts/ potato	Maximum Sprout length (cm)	Number of sprouts/ eye
Shil Bilati	100	5	5	1	3
	200	5	7	1.3	3
	300	5	9	1.3	3
	400	3	10	1.3	3
Kufri Shinduri	100	8	13	1	4
	200	5	19	1.2	4
	300	5	25	1.5	4
	400	4	27	2.2	4
Jam Alu	100	29	2	0.2	1
	200	14	3	1.5	1
	300	9	3	2	1
	400	3	5	3.5	3
Pahari Pakri	100	4	6	2.3	5
	200	2	7	2.3	5
	300	2	7	2.3	5
	400	1	18	3.1	7
Lal Pakri	100	8	1	0.5	1
	200	5	2	2	1
	300	4	2	2.5	1
	400	2	5	3.1	2
Shil Pakri	100	8	3	1	4
	200	5	3	2	4
	300	3	5	2.5	4
	400	2	7	3.5	4
	SE±	1.16	1.77	0.17	0.33
	Max	29.00	27.00	3.50	7.00
	Min	1.00	1.00	0.20	1.00
	LSD	0.07	1.21	0.39	1.06
	Level of significance	**	**	**	**

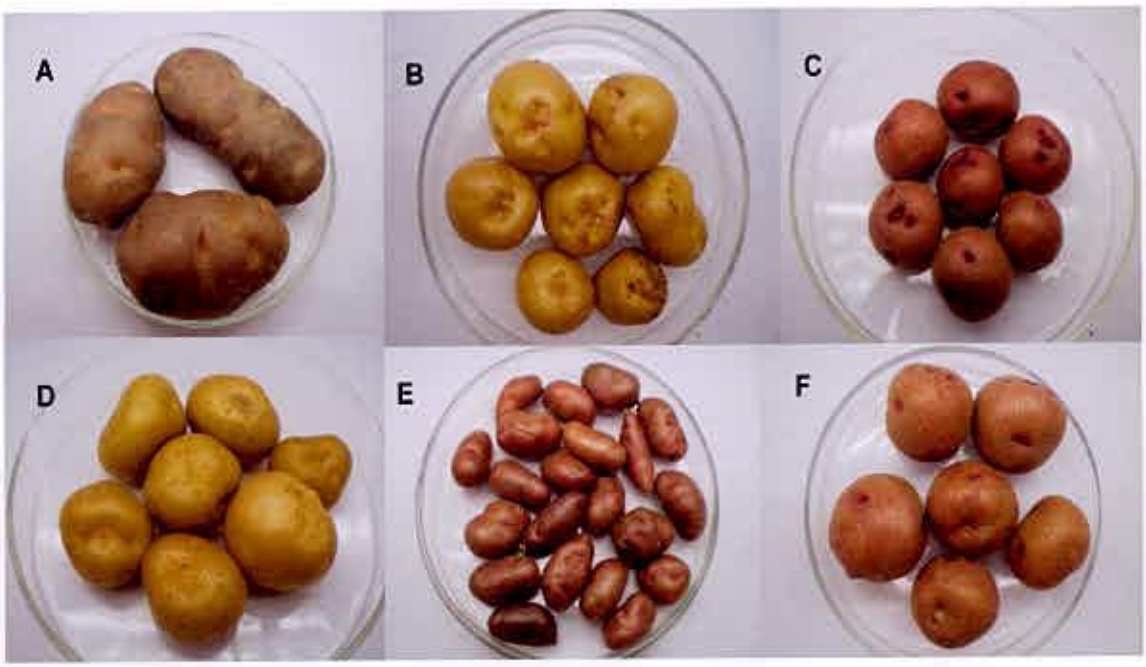

Plate 1: Primary plant materials. A. Shil Bilati , B. Kufri Shinduri, C. Lal Pakri, D. Shil Pakri, E. Jam Alu, F. Pahari Pakri

Plate 2: Virus infected potato plants grown from primary plant materials

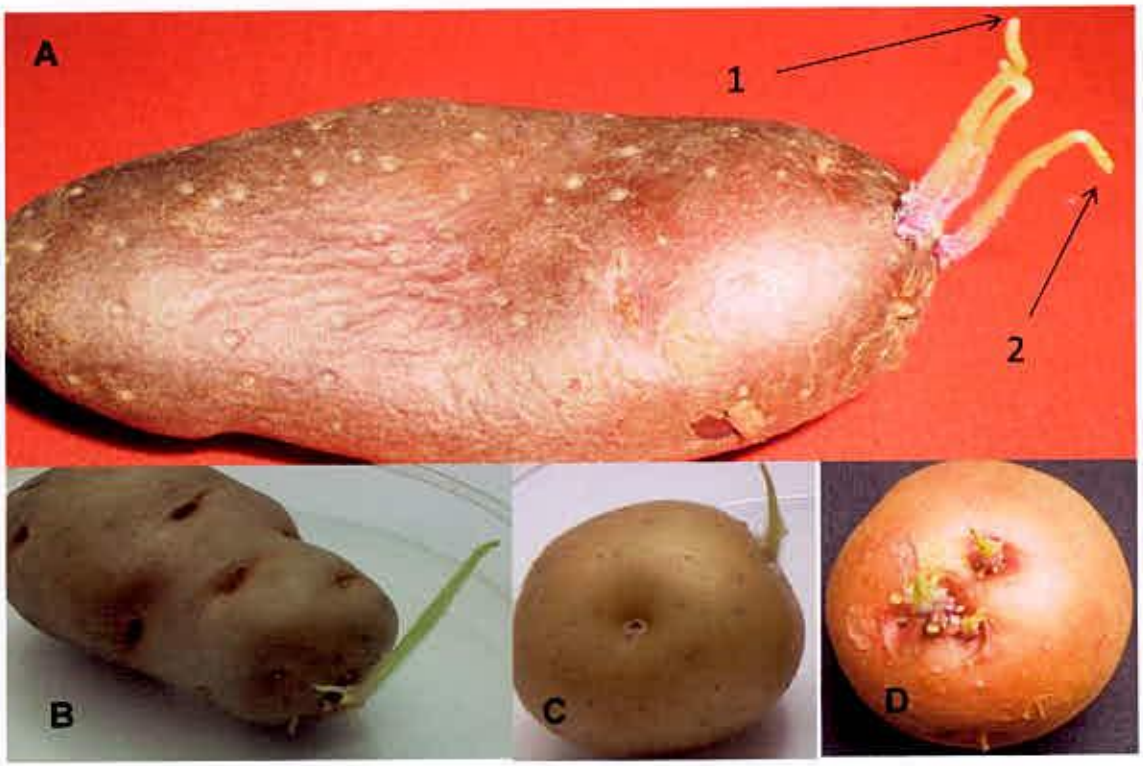

Plate 3: Sprouts of potatoes after spraying with 400 ppm GA3 treatment. Sprouts of A. Jam Alu (1 & 2 showing sprout tips), B Shil Bilati, C. Shil Pakri and D. Pahari Pakri

4.2. Experiment 2: Effect of 2, 4-D, KIN and GA3 on meristem culture, callus induction and shoot regeneration of six potato genotypes

4.2.1. Establishment of explants in the culture media

Collection of healthy, intact meristem from sprout tips and establishment of meristem was crucial part in this experiment. Collection of appropriate sized meristematic part required close observation, continuous practice with the help of microscope. After inoculation of meristem it is essential to record survival percentage for increasing efficiency in culturing meristem successfully.

4.2.2. Survival percentages after inoculation of meristem in different culture media

Meristem culture on MS media supplemented with different concentrations of GA3, 2, 4-D and Kinetin showed significant effect on survival percentage of cultured explants (Figure 1-6). It was clear that significant differences were present for survival percentage of Shil Bilati explants when treated in different hormonal treatment (Figure 1). Maximum survival percentage were observed in T0 (fresh MS media), T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/l KIN),T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN) and T4 (0.5mg/l GA3+1.0mg/l 2, 4-D+1.5mg/l KIN) and minimum (60%) was observed in T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN) and T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN).

Significant differences were observed in survival percentage of Kufri Shinduri explants when treated in different hormonal treatments (Figure 2). Maximum survival percentage was observed in T0 (fresh MS media), T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/l KIN), T4 (0.5mg/l GA3+1.0mg/l 2, 4-D+1.5mg/lKIN), T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN) and T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN). Minimum (60%) was observed in T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN).

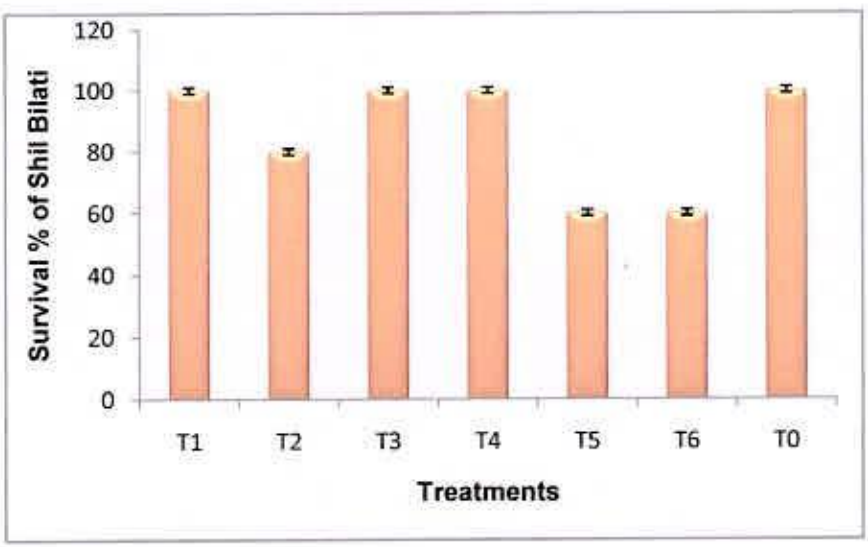

Figure 1: Effect of hormonal treatments on survival percentage of initially inoculated meristems of Shil Bilati

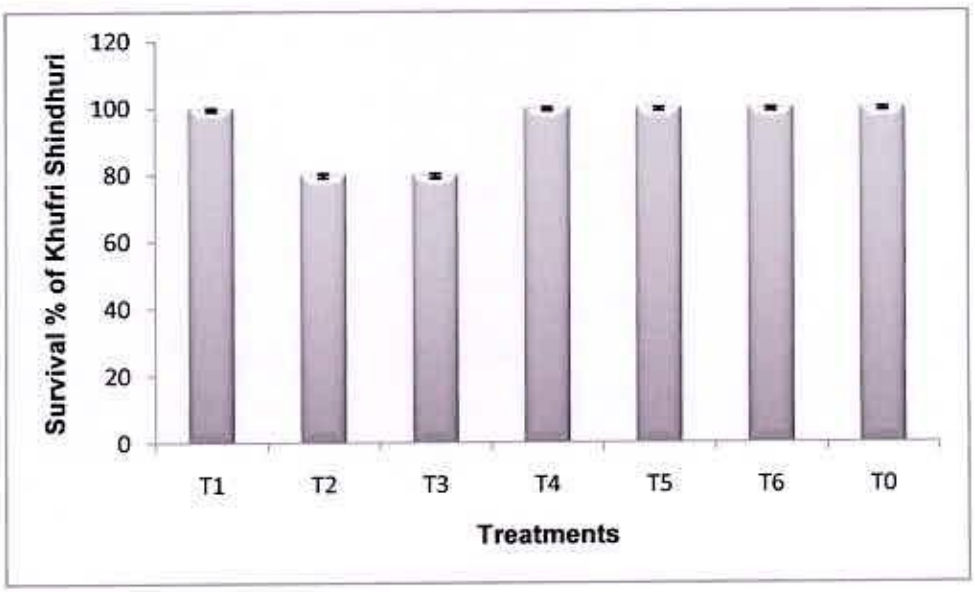

Figure 2: Effect of hormonal treatments on survival percentage of initially inoculated meristems of Kufri Shinduri

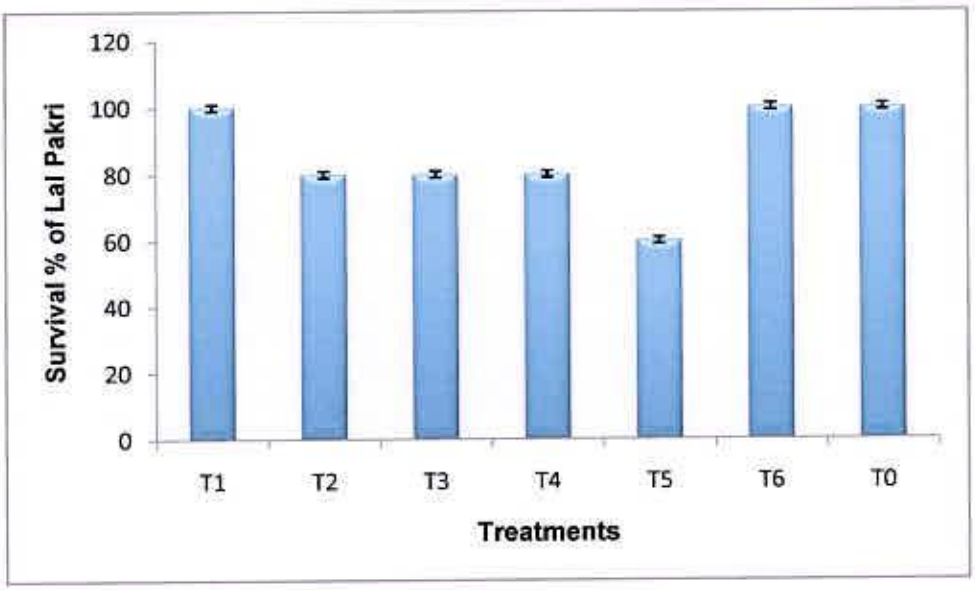

Figure 3: Effect of hormonal treatments on survival percentage of initially inoculated meristems of Lal Pakri

In the Figure 3 showing Lal Pakri explants when treated in different hormonal treatments gave maximum survival percentage in T0 (fresh MS media), T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/l KIN),and T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN). Minimum (60%) was observed in T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN).

There were significant variations observed on survival percentage of Shil Pakri explants in different treatments (Figure 4). Maximum percentage was observed in T2 (0.5mg/l GA3+0.5mg/l 2, 4-D+1.0mg/l KIN), T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN), T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN) and T0 (fresh MS media). Minimum percentage (70%) was recorded in T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/l KIN).

Variations on survival percentage in Jam Alu are presented in Figure 5. Maximum survival percentage (100 %) was observed in T2 (0.5mg/l GA3+0.5mg/l 2, 4-D+1.0mg/l KIN), T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN),T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN) ,T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN) and T0 (fresh MS media) whereas minimum survival percentage (80%) was recorded in T1 (0.5mg/l GA3+0.5mg/l 2,4-D+0.5mg/l KIN) and T4 (0.5mg/l GA3+1.0mg/l 2, 4-D+1.5mg/l KIN).

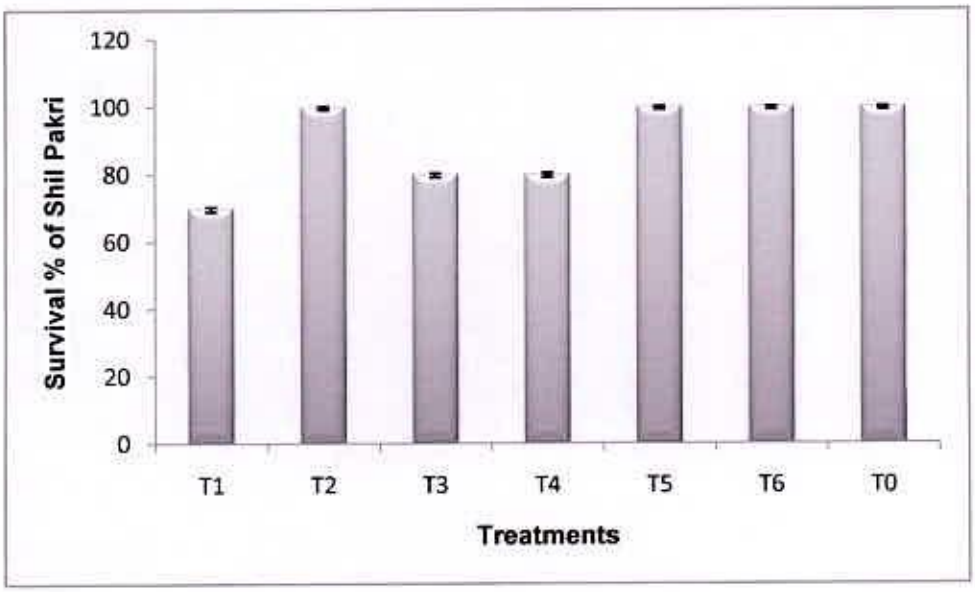

Figure 4: Effect of hormonal treatments on survival percentage of initially inoculated meristems of Shil Pakri

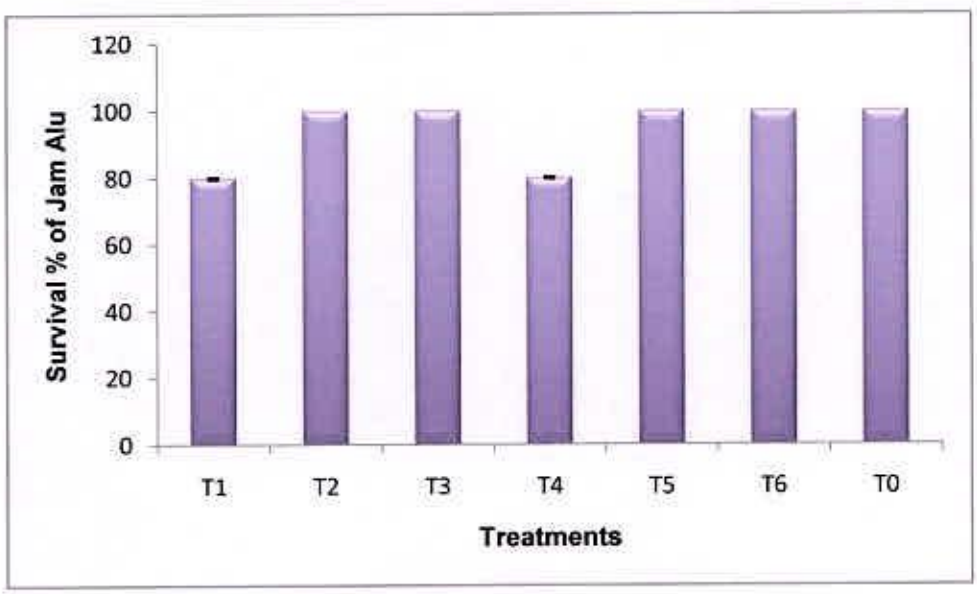

Figure 5: Effect of hormonal treatments on survival percentage of initially inoculated meristems of Jam Alu

Figure 6 showing maximum survival percentage of Pahari Pakri in T2 (0.5mg/l GA3+0.5mg/l 2, 4-D+1.0mg/l KIN), T3 (0.5mg/l GA3+0.5mg/l 2, 4-D+1.0mg/l KIN), T5 (0.5mg/l GA3+1.0mg/l 2,4-D+ 1.0mg/l KIN), T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN) and T0 (fresh MS media). Minimum percentage (40%) was observed in T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/l KIN).

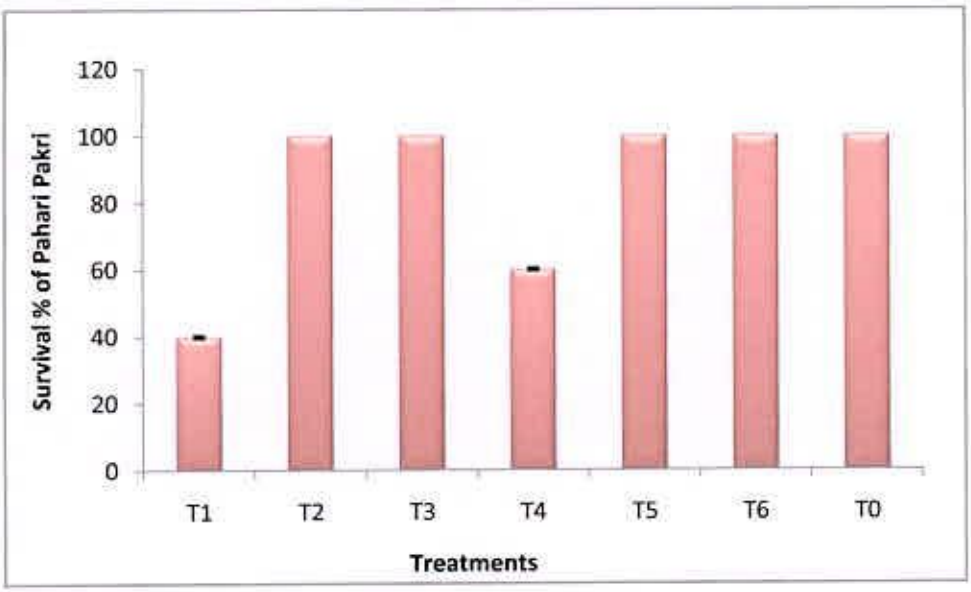

Figure 6: Effect of hormonal treatments on survival percentage of initially inoculated meristems of Pahari Pakri

4.2.3. Days to callus initiation:

Meristems (Plate 4) of six potato genotypes were isolated from freshly grown sprouts and cultured in different combinations of GA3, 2, 4-D and Kinetin. Recorded results on effects of different hormonal combinations on callus initiation and proliferation of different genotypes is presented in Table 2, 3 and 4.

The results of major effect of potato genotypes on the number of days required for callus initiation have been presented in Table 2. The number of days required for callus initiation varied significantly among six potato genotypes. The maximum number of days required for callus initiation was recorded in Shil Pakri (7.68 days) where as minimum number of days required for callus initiation was recorded in Shil Bilati (3.66 days).

Table 2: The Effect of six potato genotypes on days to callus initiation and proliferation

Potato genotypes	Days to callus initiation	Callus Size (cm)
Shil Bilati	3.66	0.29
Kufri Shinduri	5.17	0.66
Lal Pakri	6.67	0.40
Shil Pakri	7.68	0.33
Jam Alu	7.17	0.39
Pahari Pakri	6.50	0.32
SE±	2.50	0.16
Max	7.68	0.66
Min	3.66	0.29
LSD	0.78	0.05
Level of significance	**	**

The Effect of different hormonal combinations on days to callus initiation and proliferation has been presented in Table 3. Maximum number of days required (9.17 days) for callus initiation was recorded at T6 hormonal combinations (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN) and minimum number of days required (3.33 days) for callus initiation was noticed at T3 hormonal combinations (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN). No callus was initiated when explants cultured in fresh MS media (Table-3).

Table 3: Effect of different hormonal treatments on days to callus initiation and proliferation

Treatment	Days to callus initiation	Callus Size
T1	5.83	0.31
T2	4.33	0.36
T3	3.33	0.42
T4	6.50	0.54
T5	7.86	0.44
T6	9.17	0.31
T0	-	-
SE±	1.19	0.07
Max	9.17	0.54
Min	3.33	0.31
LSD	0.78	0.05
Level of significance	**	**

The potato genotypes and different levels of hormonal treatments showed significant interaction in relation to the number of days to callus initiation (Table 4). The maximum days (15days) required for callus initiation was observed in Shil Pakri cultured in T5 (0.5 mg/l GA3 +1 mg/l 2,4-D+ 0.5mg/l KIN) and in the same genotype that is Shil Pakri cultured in T6 (0.5 mg/l GA3 +1 mg/l 2,4-D+ 1 mg/l KIN). The minimum day (1 day) required for callus initiation was observed in Shil Bilati when cultured in T4 (0.5 mg/l GA3 +1 mg/l 2, 4-D+ 1.5mg/l KIN).

4.2.4. Size of callus

There were significant differences in size of callus among these potato genotypes (Table 2). Maximum callus size was recorded (0.66 cm) in Kufri Shinduri whereas minimum callus size was recorded (0.29 cm) in Shil Bilati.

It is clear from data shown in Table 3 and picture shown in Plate 7 and 8 that among different hormonal treatments T4 (0.5 mg/l GA3 +1 mg/l 2, 4-D+ 1.5mg/l KIN) produced maximum size of callus (0.54 cm) whereas minimum size of callus (0.31 cm) was observed in T1 (0.5 mg/l GA3 +0.5 mg/l 2, 4-D+ 0.5 mg/l KIN). No callus was formed when explants were cultured in fresh media with no hormone (T0).

Various hormonal treatments showed significant variations on callus size when explants were inoculated in various hormonal treatments due to interaction effect of different potato genotypes and hormonal treatments on size of callus (Table 4). Maximum size of callus was observed (0.82 cm) when meristem of Kufri Shinduri (plate 8) was inoculated in T4 (0.5 mg/l GA3 +1.0 mg/l 2, 4-D+ 1.5 mg/l KIN) whereas minimum size of callus was recorded (0.17 cm) when meristem of Shil Bilati was inoculated in T1 (0.5 mg/l GA3 +0.5 mg/l 2, 4-D+ 0.5 mg/l KIN). No callus was initiated in T0 (fresh MS media).

Table 4: Effect of different genotypes and hormonal treatments on callus initiation and proliferation

Potato genotype	Treatment	Days to callus initiation	Callus Size (cm)
Shil Bilati	T1	9.00	0.17
Kufri Shinduri	T1	7.00	0.39
Lal Pakri	T1	8.00	0.34
Shil Pakri	T1	5.00	0.24
Jam Alu	T1	6.00	0.30
Pahari Pakri	T1	5.00	0.28
Shil Bilati	T2	4.00	0.31
Kufri Shinduri	T2	3.00	0.61
Lal Pakri	T2	5.00	0.42
Shil Pakri	T2	3.00	0.23
Jam Alu	T2	5.00	0.28
Pahari Pakri	T2	6.00	0.28
Shil Bilati	T3	2.00	0.27
Kufri Shinduri	T3	5.00	0.73
Lal Pakri	T3	3.00	0.50
Shil Pakri	T3	2.00	0.58
Jam Alu	T3	3.00	0.71
Pahari Pakri	T3	5.00	0.37
Shil Bilati	T4	1.00	0.41
Kufri Shinduri	T4	5.00	0.82
Lal Pakri	T4	5.00	0.28
Shil Pakri	T4	9.00	0.38
Jam Alu	T4	10.00	0.40
Pahari Pakri	T4	9.00	0.35
Shil Bilati	T5	3.00	0.23
Kufri Shinduri	T5	5.00	0.80
Lal Pakri	T5	10.00	0.54

Table 4 (cont'd)

Shil Pakri	T5	15.00	0.20
Jam Alu	T5	10.00	0.46
Pahari Pakri	T5	7.00	0.34
Shil Bilati	T6	9.00	0.18
Kufri Shinduri	T6	6.00	0.63
Lal Pakri	T6	9.00	0.29
Shil Pakri	T6	15.00	0.31
Jam Alu	T6	4.00	0.31
Pahari Pakri	T6	7.00	0.28
Shil Bilati	T0	-	-
Khfri Shinduri	T0	-	-
Lal Pakri	T0	-	-
Shil Pakri	T0	-	-
Jam Alu	T0	-	-
Pahari Pakri	T0	-	-
	SE±	0.48	0.03
	Max	15.00	0.82
	Min	1.00	0.17
	LSD	1.91	0.13
	Level of significance	**	**

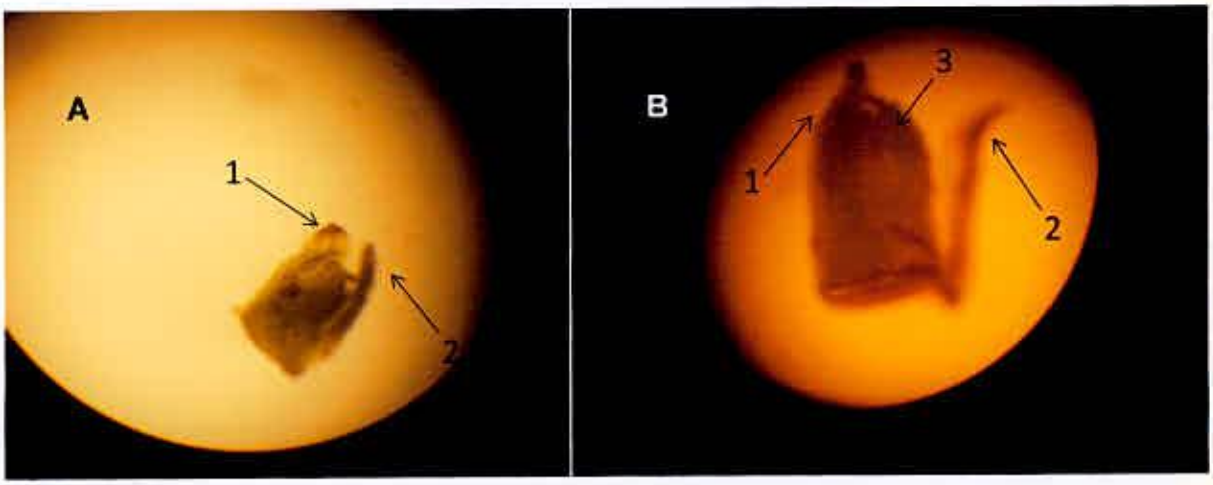

Plate 4: **Shoot tip excised from potato sprouts under 50x magnification binocular microscope. A. Meristem surrounded by numerous leaf promordia (1 & 2 showing leaf primordia), B. Shoot tip including meristem surrounded by two (1 &2) leaf primordia (3 showing meristem tip)**

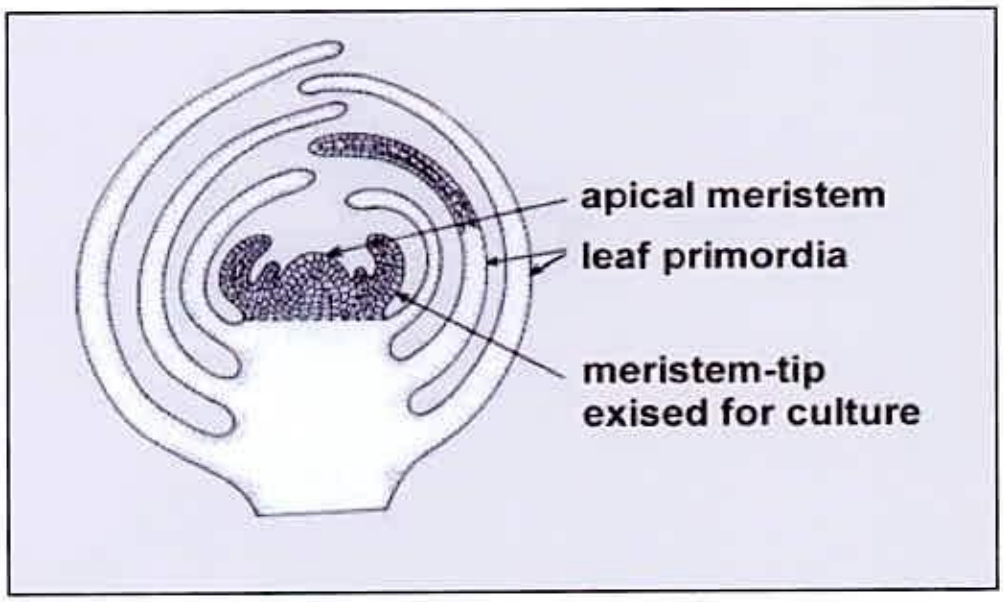

Figure 7: Region showing meristem tip excised for culture

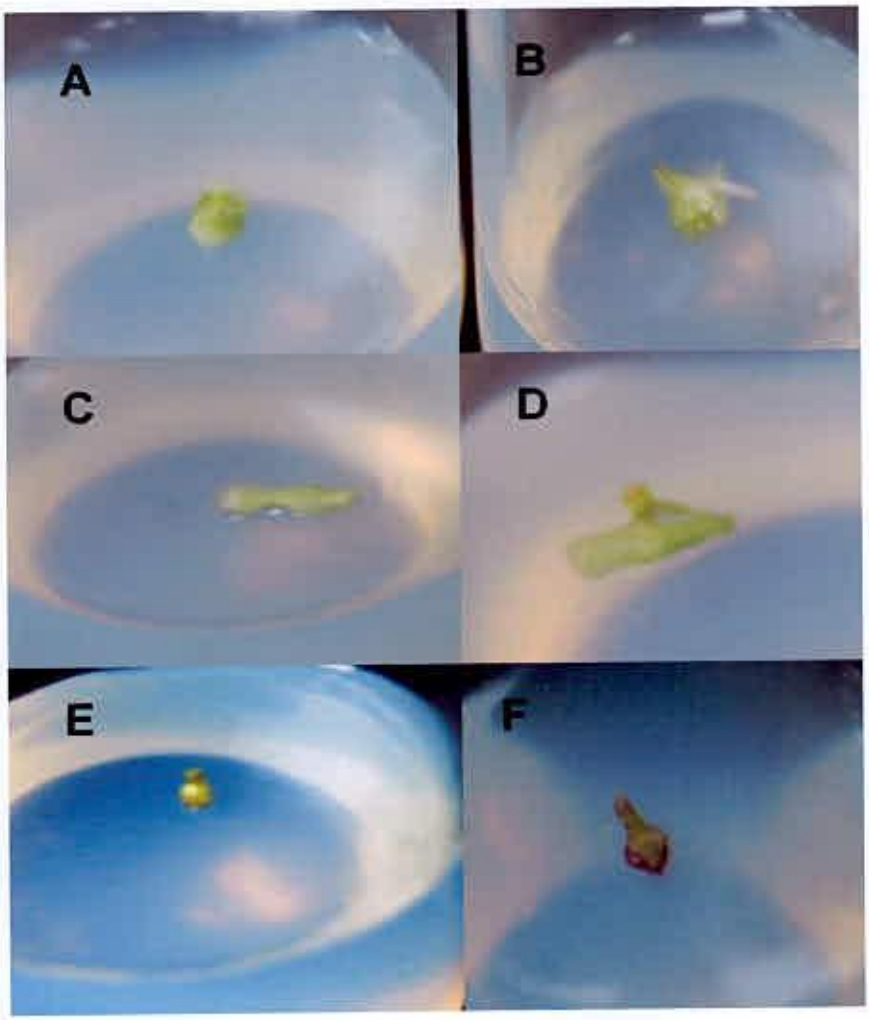

Plate 5: Meristems of Shil Bilati, Kufri Shinduri and Lal Pakri in treatment T1 (0.5mg/l GA3 + 0.5 mg/l 2, 4-D + 0.5 mg/l Kin). A. 7 days old meristem of Shil Bilati , B. 14 days old meristem of Shil Bilati, C. 7 days old meristem of Kufri Shinduri, D. 14 days old meristem of Kufri Shinduri, E. 7 days old meristem of Lal Pakri , F. 14 days old meristem of Lal Pakri

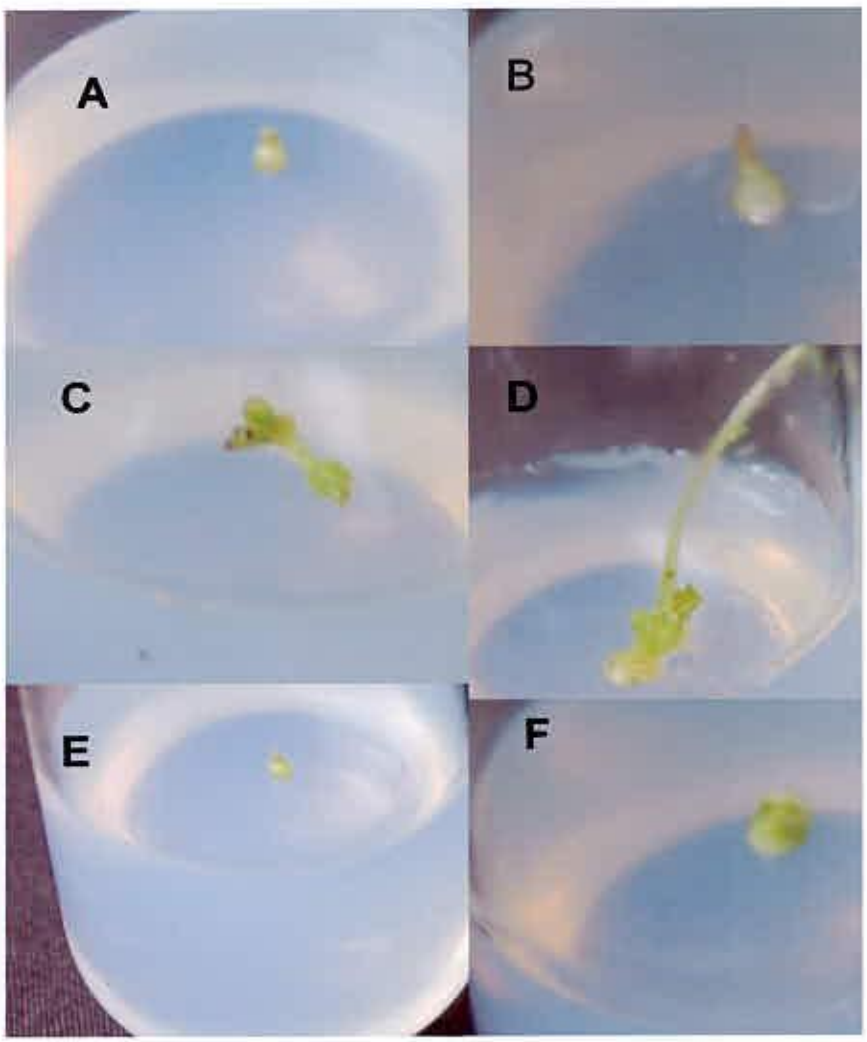

Plate 6: Meristems of Shil Pakri, Jam Alu and Pahari Pakri in treatement T1 (0.5mg/l GA3 + 0.5 mg/l 2, 4-D + 0.5 mg/l Kin). A. 7 days old meristem of Shil Pakri, B. 14 days old meristem of Shil Pakri, C. 7 days old meristem of Jam Alu, D. 14 days old meristem of Jam Alu, E. 7 days old meristem of Pahari Pakri , F. 14 days old meristem of Pahari Pakri

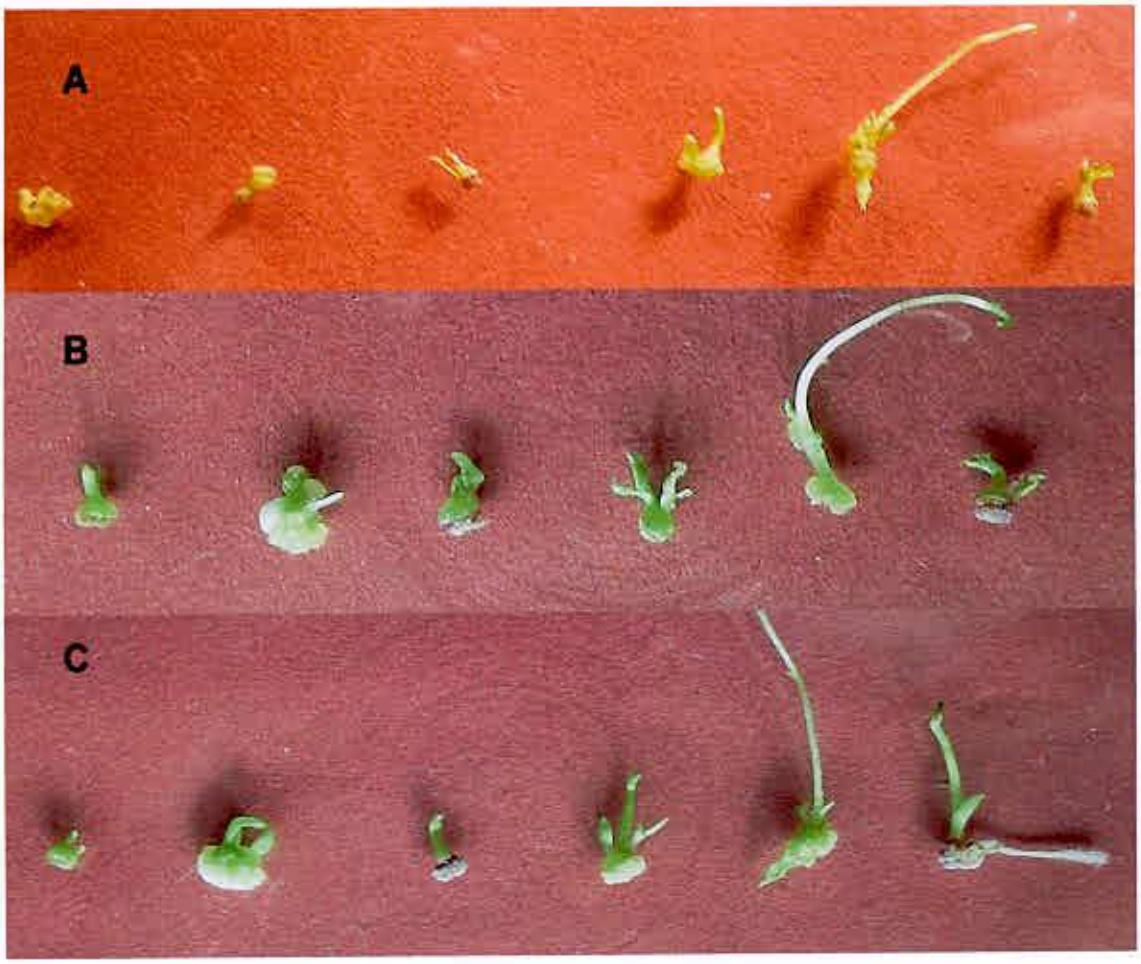

Plate 7: 21 days old meristem in T1 (A), T2 (B) and T3 (C) of Shil Bilati, Kufri Shinduri, Lal Pakri, Shil Pakri, Jam Alu and Pahari Pakri (from left to right). A. Meristem in T1 (0.5mg/l GA3+0.5mg/l 2,4-D+0.5mg/l KIN), B. T2 (0.5mg/l GA3+0.5mg/l 2,4-D+1.0mg/l KIN), C. T3. (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN

Plate 8: 21 days old meristem in T4 (A), T5 (B) and T6 (C) of Shil Bilati, Kufri Shinduri, Lal Pakri, Shil Pakri, Jam Alu and Pahari Pakri (from left to right). A. Meristem in T4 (0.5mg/l GA3+1.0mg/l 2,4-D+1.5mg/lKIN), B. T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN), C. T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN)

4.2.5. Callus texture and callus color

After inoculation in culture media at the cut edge of meristem initially a creamy white appearance occurred. Gradually there developed different colors in different potato genotypes (Plate 9). 30 days after inoculation of meristem, cultured meristem produced callus which was examined for determining texture of callus and data was recorded (Table 5).

Table 5: Texture and color of six potato genotypes 30 DAI

Variety	Callus color	Callus texture
Shil Bilati	Greenish	Friable
Kufri Shinduri	Whitish green	Non friable
Lal Pakri	Reddish	Friable
Shil Pakri	Yellowish green	Friable
Jam Alu	Yellowish green	Non friable
Pahari Pakri	Reddish	Friable

Plate 9: 30 days old Callus of six potato genotypes in T4 (0.5 mg/l GA3+ 1.0 mg/l 2, 4- D + 1.5 mg/l KIN). A. Shil Bilati, B. Kufri Shinduri, C. Lal Pakri, D. Shil Pakri, E. Jam Alu, and F. Pahari Pakri

4.2.6. Regeneration of single shoot from callus induced by meristem culture

It is highly expected to get multiple shoot through tissue culture practices. But multiple shoot formation is impossible without forming a single shoot so single shoot regeneration is a preliminary event of *in vitro* regeneration practice. During callusing of explants data was recorded when single shoot was regenerated from callus. Very slow growth of shoot was observed during the experiment.

4.2.7. Days to shoot initiation

Effect of various potato genotypes on shoot initiation and length of shoot has been presented in Table 6. Maximum days required (9.14 days) for shoot initiation was recorded in Lal Pakri and a minimum day required (5.33 days) for shoot initiation was recorded in Shil Bilati.

Table 7 representing effect of different treatments on days required for shoot initiation revealed that MS media containing T6 treatment (0.5 mg/l GA3 + 1.0 mg/l 2, 4-D+ 1.0mg/l KIN) took maximum days (14.50 days) to initiate single shoot from callus induced from meristem and medium containing T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 1.5 mg/l KIN) took minimum days required (5.13 days) for shoot initiation.

Combined effect of different treatment and potato genotypes on days required for shoot initiation (Table 8) revealed that Lal Pakri took maximum days to initiate shoot (19 days) when cultured in media containing T2 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 1.0 mg/l KIN) whereas Kufri Shinduri, Lal Pakri and Shil Pakri took minimum days for shoot initiation (2 days) when cultured in T0 (MS media) and T2 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 0.5 mg/l KIN) and Kufri Shinduri showed no response for shoot initiation when cultured in media containing T2 treatment (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 0.5 mg/l KIN). No significant change was observed when Shil Bilati and Shil Pakri was cultured in medium containing T6 (0.5 mg/l GA3 + 1.0 mg/l 2, 4-D+ 1.0mg/l KIN) and Pahari Pakri was cultured in medium containing T5 (0.5 mg/l GA3 + 1.0 mg/l 2, 4-D+ 0.5 mg/l KIN).

4.2.8. Days to root initiation

Days required for rooting was found non-significant in this experiment.

4.2.9. Shoot length

The length of regenerated shoot per callus varied significantly among different potato genotypes at 14, 21 and 30 DAI. The highest shoot length (2.41 cm) was recorded in Shil Pakri 30 DAI and the shortest shoot length (0.27 cm) was recorded in Shil Bilati 30 DAI (Table 6 and plate 10).

Table 6: Effect of different potato genotypes on regeneration of single shoot/ callus

Potato genotype	Days to shoot initiation	Shoot length (cm)			Days to root initiation
		14 DAI	21 DAI	30 DAI	
Shil Bilati	5.33	0.22	0.25	0.27	-
Kufri Shinduri	8.74	0.19	0.36	1.07	19.17
Lal Pakri	9.14	0.35	0.55	1.60	15.77
Shil Pakri	5.64	0.52	0.75	2.41	11.15
Jam Alu	7.58	0.58	0.59	1.38	-
Pahari Pakri	7.00	0.35	0.47	1.41	17.61
SE±	3.36	0.13	0.20	0.61	6.17
Max	9.14	0.58	0.75	2.41	19.17
Min	5.33	0.19	0.25	0.27	17.61
LSD	0.91	0.11	0.14	0.54	-
Level of significance	**	**	**	**	NS

Shoot length/ callus were significantly influenced by different concentrations of GA3, 2, 4-D and KIN at 14, 21 and 30 DAI (Table 7). The highest shoot length (2.42 cm) was found in T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 1.5 mg/l KIN) at 30 DAI whereas lowest shoot length (0.53cm) was recorded in T2 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 0.5 mg/l KIN).

Table 7: Effect of different hormonal treatments on regeneration of single shoot/ callus

Treatment	Days to shoot initiation	Shoot length (cm)			Days to root initiation
		14 DAI	21 DAI	30 DAI	
T1	5.60	0.29	0.50	0.77	-
T2	5.63	0.36	0.49	0.53	16.47
T3	5.13	0.39	0.52	2.42	7.60
T4	10.27	0.26	0.42	2.37	21.43
T5	11.19	0.44	0.51	0.62	20.00
T6	14.50	0.15	0.50	0.61	21.50
T0	2.67	0.55	0.55	0.57	-
SE±	1.99	0.12	0.15	0.73	2.71
Max	14.50	0.44	0.52	2.42	21.50
Min	5.13	0.15	0.42	0.53	7.60
LSD	1.02	0.12	0.15	0.59	
Level of significance	**	**	**	**	NS

Table 8: Effect of different hormonal treatments and potato genotypes on regeneration of single shoot/ callus

Potato genotype	Treatment	Days to shoot initiation	Shoot length (cm)			Days to root initiation
			14 DAI	21 DAI	30 DAI	
Shil Bilati	T1	--	-	-	-	-
Kufri Shinduri	T1	7.60	0.14	0.20	0.20	-
Lal Pakri	T1	10.00	0.30	0.82	1.08	-
Shil Pakri	T1	6.00	0.36	0.50	0.62	-
Jam Alu	T1	4.00	0.56	0.98	1.48	-
Pahari Pakri	T1	6.00	0.40	0.50	1.26	-
Shil Bilati	T2	-	-	-	-	-
Kufri Shinduri	T2	-	-	-	-	21.00
Lal Pakri	T2	19.00	-	0.20	0.20	20.00
Shil Pakri	T2	4.40	0.64	0.70	0.70	10.00
Jam Alu	T2	5.00	1.08	1.58	1.70	-
Pahari Pakri	T2	5.40	0.42	0.46	0.56	14.00
Shil Bilati	T3	4.00	0.30	0.34	0.34	-
Kufri Shinduri	T3	13.00	0.16	0.26	1.68	14.00
Lal Pakri	T3	5.00	0.48	0.60	2.62	9.00
Shil Pakri	T3	3.00	0.50	0.60	5.88	5.00
Jam Alu	T3	2.20	0.54	0.82	1.94	0.00
Pahari Pakri	T3	3.60	0.34	0.48	2.06	10.00

Table 8 (cont'd)

Shil Bilati	T4	12.00	0.13	0.24	0.36	-
Kufri Shinduri	T4	14.60	0.00	0.36	2.60	22.00
Lal Pakri	T4	4.00	0.28	0.50	3.26	-
Shil Pakri	T4	9.00	0.38	0.50	3.52	20.00
Jam Alu	T4	10.00	0.44	0.52	1.98	-
Pahari Pakri	T4	12.00	0.32	0.38	2.48	22.00
Shil Bilati	T5	13.00	0.30	0.30	0.34	-
Kufri Shinduri	T5	9.00	0.50	0.58	0.64	20.00
Lal Pakri	T5	10.00	0.36	0.56	0.96	-
Shil Pakri	T5	12.00	0.74	0.74	0.74	-
Jam Alu	T5	13.00	0.20	0.30	0.30	-
Pahari Pakri	T5	-	-	-	-	-
Shil Bilati	T6	-	-	-	-	-
Kufri Shinduri	T6	15.00	0.00	0.60	0.60	-
Lal Pakri	T6	14.00	0.30	0.50	0.70	20.00
Shil Pakri	T6	-	-	-	-	-
Jam Alu	T6	17.00	-	0.40	0.50	-
Pahari Pakri	T6	12.00	0.14	0.50	0.64	23.00
Shil Bilati	T0	3.00	0.60	0.60	0.60	-
Kufri Shinduri	T0	2.00	0.50	0.50	0.60	-
Lal Pakri	T0	2.00	0.70	0.70	0.70	
Shil Pakri	T0	2.00	0.50	0.50	0.50	-
Jam Alu	T0	4.00	0.50	0.50	0.50	-
Pahari Pakri	T0	3.00	0.50	0.50	0.50	-
	SE±	0.81	0.05	0.06	0.22	1.11
	Max	19.00	1.08	1.58	5.88	23.00
	Min	2.00	0.13	0.20	0.20	1.00
	LSD	2.50	0.29	0.37	1.15	
	Level of significance	**	**	**	**	NS

The combined effect of potato genotypes and different concentrations of hormones showed variations at different days on shoot length (Table 8). The maximum shoot length (5.88 cm) was recorded in Shil Pakri in T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 1.5 mg/l KIN) 30 DAI and minimum shoot length (0.20cm) was observed in Lal Pakri in T2(0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 0.5 mg/l KIN) and Kufri Shinduri in T1 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 1.0 mg/l KIN) 30 DAI. T3 was found most effective treatment among all treatments to initiate and regenerate single shoot from callus induced from meristem and Shil Pakri was the best genotype in response to hormonal treatment T3. Shil Bilati in T2 (0.5mg/l GA3+0.5mg/l 2, 4-D+1.0mg/l KIN) and T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN), Kufri Shinduri in T2 (0.5mg/l GA3+0.5mg/l 2, 4-D+1.0mg/l KIN) and Pahari Pakri In T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0mg/l KIN) showed no interaction effect in shoot length.

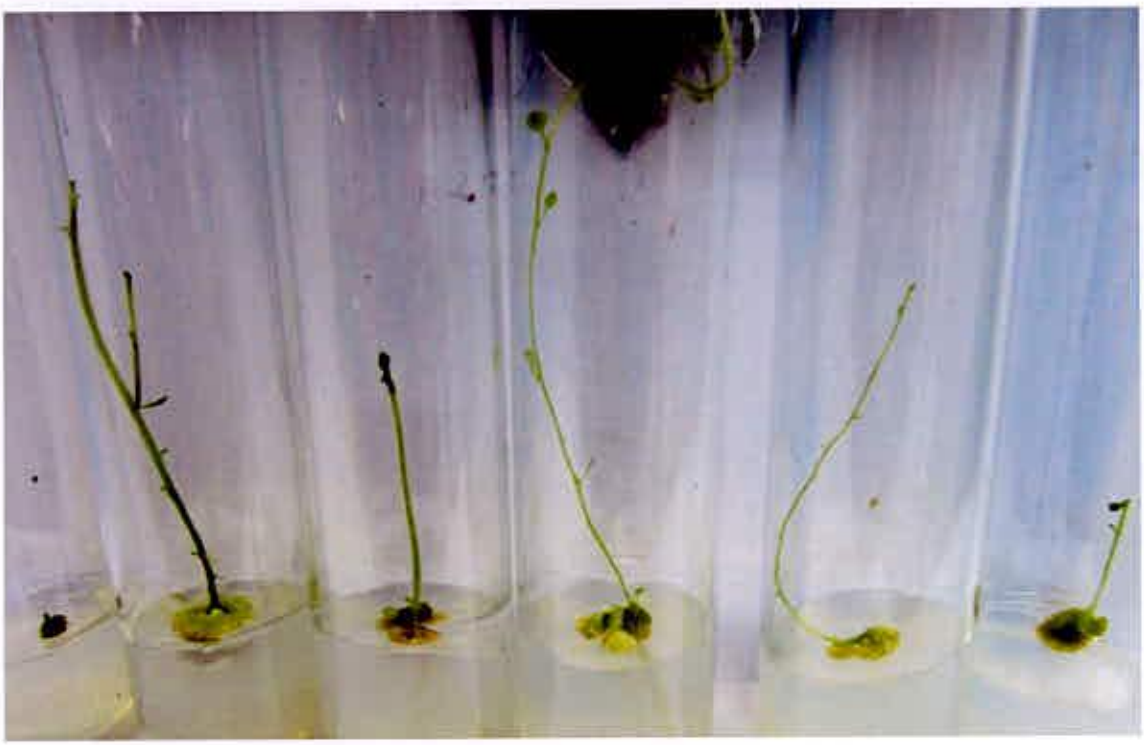

Plate 10 : 30 day's old meristem of Shil Bilati , Kufri Shinduri, Lal Pakri, Shil Pakri , Jam Alu and Pahari Pakri respectively in T3 (0.5 mg/l +0.5 mg/l 2, 4-D + 1.5 mg/l KIN). Highest shoot lenght in Shil Pakri and lowest in Shil Bilati

Plate 11: 30 days old meristem culture treated in T3 (0.5 mg/l GA3+0.5 mg/l 2, 4-D+ 1.5 mg/l KIN). A. Shila Bilati, B. Kufri Shinduri, C. Lal Pakri, D. Shil Pakri (larger view of plate 10)

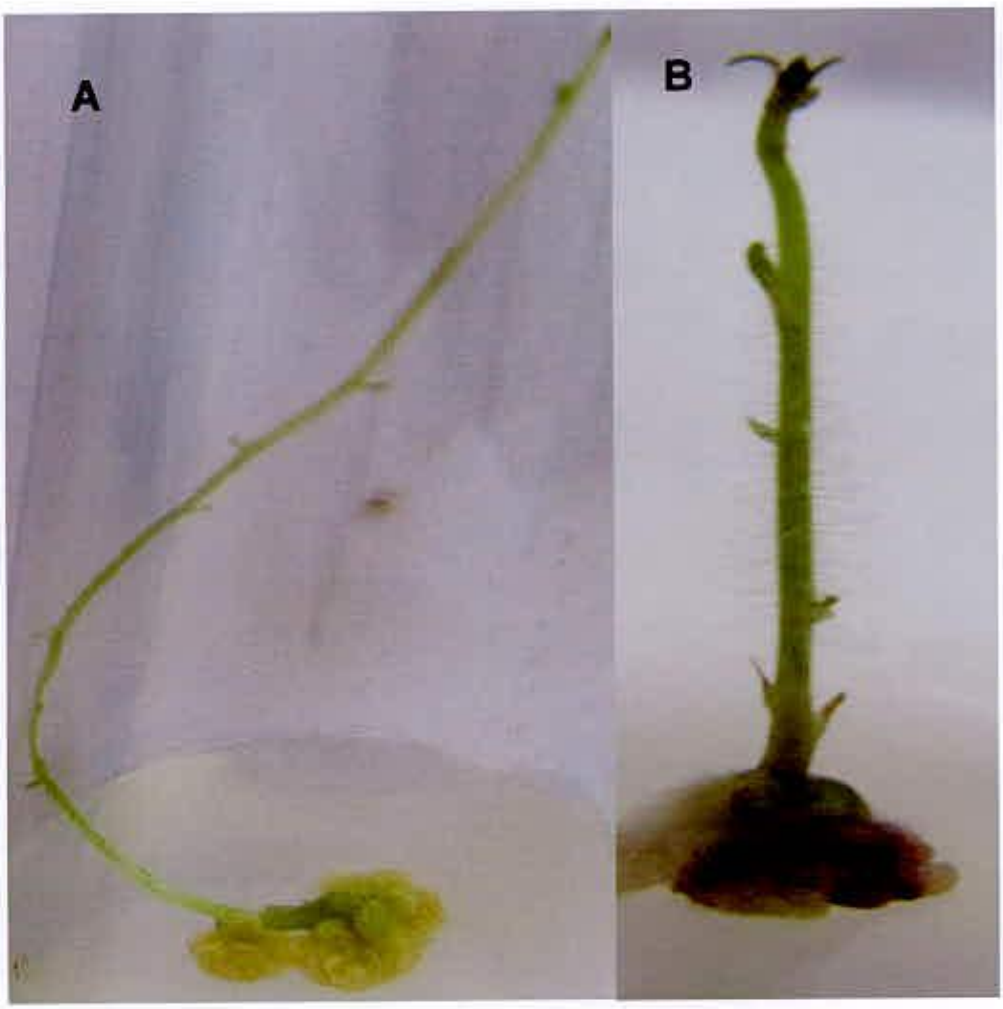

Plate 12: 30 days old meristem culture treated in T3 (0.5 mg/l GA3+0.5 mg/l 2, 4-D+ 1.5 mg/l KIN). A. Jam Alu, B. Pahari Pakri (larger view of plate 10)

4.3. Experiment 3: Effect of 21 days old subculture performance under different GA3 hormonal treatments

Multiplication of shoot for *in vitro* regeneration and plant establishment is essential part of meristem culture. Since single shoot regenerated from callus by meristem culture shows slow growth of shoot and plantlet. Three levels of media (Fresh media, 0.5 mg/l and 1.5 mg/l GA3) were used to find out best hormone for rapid multiplication of meristem culture derived shoot from experiment 2. The subculture performance of 21 days old plantlet is being discussed under following heads.

4.3.1. Plant height

There were significant variations recorded among different potato genotypes on plant height presented in Table 9. Maximum plant height was recorded (8.96 cm) in Shil Pakri and minimum plant height (0.67 cm) was recorded in Shil Bilati.

The effect of three different treatments was observed and significant variations on plant height have been presented in Table 10. The maximum plant height was recorded (5.68 cm) in T2 (0.5 mg/l GA3) and minimum plant height was recorded (4.10 cm) in T1 (Fresh MS media).

Different cultivars and concentration of GA3 influenced the plant height per explants (Table 11). Maximum plant height was observed (9.50 cm) in Shil Pakri when cultured in T2 (0.5 mg/l GA3) whereas minimum plant height (0.27 cm) was observed in Shil Bilati when cultured in T1 (Fresh media).

4.3.2. Number of branches per plant

There were significant variations recorded among different potato genotypes presented in Table 10. Maximum number of branches per plant was recorded (2.78) in Shil Pakri and minimum number of branches (1.14) was recorded in Jam Alu. No branch was observed in Shil Bilati.

The effect of three different treatments was observed and data was recorded on number of branches/ plantlet which have been presented in Table 11. The maximum number of branches/ plantlet was recorded (2.60) in T2 (0.5 mg/l GA3) and minimum number of branches/ plantlet was recorded (1) in T1 (Fresh media).

Different potato genotypes and concentration of GA3 influenced the number of branches/ plantlet (Table 11). Maximum number of branches/ plantlet was observed (5) in Shil Pakri when cultured in T2 (0.5 mg/l GA3) whereas minimum number of branches/ plantlet (1) was observed in Jam Alu when cultured in T1 (Fresh media) and T2 (0.5 mg/l GA3) as well as in Shil Pakri when cultured in T1 (Fresh media). No response was recorded in cv. Shil Bilati when cultured in T1 (Fresh media), T2 (0.5 mg/l GA3), T3 (1.5 mg/l GA3) and in Kufri Shinduri when cultured in T1 (Fresh media). Also no significant response was observed in Pahari Pakri in T1 (Fresh media).

Table 9: Effect of 21 days old subculture performance on different potato genotypes for rapid multiplication of single shoot derived from meristem culture

Potato genotype	Plant height (cm)	Number of branches / plantlet	Numbe r of roots/ plantlet	Root length (cm)	Number of leaves/ plantlet
Shil Bilati	0.67	-	-	-	-
Kufri Shinduri	2.12	1.50	1.83	9.33	5.40
Lal Pakri	5.32	2.17	4.11	8.77	12.63
Shil Pakri	8.96	2.78	4.33	8.77	22.56
Jam Alu	4.14	1.14	2.00	3.28	3.89
Pahari Pakri	5.61	2.60	2.25	7.41	11.89
SE±	1.25	0.57	0.75	1.60	3.76
Max	8.96	2.78	4.33	9.33	22.56
Min	0.67	1.14	1.83	3.28	3.89
LSD	1.37	0.72	1.16	2.57	4.06
Level of significance	**	**	**	**	**

4.3.3. Number of roots/ plantlet

Significant variations were recorded among different potato genotypes on number of roots/ plantlet presented in Table 10. Maximum number of roots/ plantlet (4.33) was recorded in Shil Pakri and minimum number of roots/ plantlet (1.83) was recorded in Kufri Shinduri. No root formation was observed in cv. Shil Bilati.

The effect of three different treatments was observed showed significant variations on number of roots/ plantlet which have been presented in Table 11. The maximum number of roots/ plantlet (3.89) was recorded in T1 (Fresh media) and minimum number of roots/ plantlet (2.67) was recorded in T3 (1.5 mg/l GA3).

Different cultivars and concentration of GA3 influenced the number of roots/ plantlet per explants (Table 11). Maximum number of roots/ plantlet (6) was observed in Shil Pakri when cultured in T1 (fresh media) whereas minimum number of roots/ plantlet (1) was observed in Jam Alu when cultured in T1 (Fresh media) and T2 (0.5 mg/l GA3). No response was recorded in Shil Bilati when cultured in T1 (Fresh media), T2 (0.5 mg/l GA3), T3 (1.5 mg/l GA3) and in Kufri Shinduri when cultured in T1 (Fresh media).

Table 10: Effect of different GA3 hormonal treatments for rapid multiplication of single shoot derived from meristem culture

Treatment	Plant height (cm)	Number of branches/ plantlet	Number of roots/ plantlet	Root length (cm)	Number of leaves/ plantlet
T1	4.10	1.00	3.89	7.65	8.45
T2	5.68	2.60	2.93	8.74	14.86
T3	5.06	1.79	2.67	7.21	11.47
SE±	1.76	0.80	1.06	2.26	5.32
Max	5.68	2.60	3.89	8.74	14.86
Min	4.10	1.00	2.67	7.21	8.45
LSD	0.97	0.51	0.82	1.82	2.87
Level of significance	**	**	**	**	**

4.3.4. Root length

Root length also showed significant variations among different potato genotypes as data presented in Table 9. Maximum length of root (9.33 cm) was recorded in Kufri Shinduri and minimum length of root (3.28 cm) was recorded in Jam Alu. No root formation was observed in Shil Bilati.

The effect of three different treatments was observed showing significant variations on root length have been presented in Table 10. The maximum length of root (8.74 cm) was recorded in T2 (0.5 mg/l GA3) and minimum length of root (7.21 cm) was recorded in T3 (1.5 mg/l GA3).

Table 11: Effect of 21 days old subculture performance and different GA3 hormonal treatments for rapid multiplication of single shoot derived from meristem culture

Potato genotype	Treatment	Plant height (cm)	Number of branches/ plantlet	Number of roots/ plantlet	Root length (cm)	Number of leaves/ plantlet
Shil Bilati	T1	-	-	-	-	-
	T2	0.27	-	-	-	-
	T3	1.07	-	-	-	-
Kufri Shinduri	T1	-	-	-	-	-
	T2	2.10	1.33	1.67	9.27	8.00
	T3	2.13	1.67	2.00	9.40	3.67
Lal Pakri	T1	4.37	-	4.00	6.83	3.50
	T2	5.40	2.33	5.00	11.50	15.00
	T3	6.20	2.00	3.33	7.97	16.33
Shil Pakri	T1	8.83	1.00	6.00	11.47	14.67
	T2	9.50	5.00	4.33	5.67	33.67
	T3	8.53	2.33	2.67	9.17	19.33
Jam Alu	T1	5.43	1.00	1.00	1.00	4.33
	T2	2.23	1.00	1.00	1.00	1.33
	T3	4.77	1.33	3.33	5.57	6.00
Pahari Pakri	T1	3.40	-	2.00	7.50	9.67
	T2	5.77	3.33	2.67	10.83	14.00
	T3	7.67	1.50	2.00	3.93	12.00
	SE±	0.72	0.33	0.43	0.92	2.17
	Max	9.50	5.00	6.00	11.50	33.67
	Min	0.27	1.00	1.00	1.00	1.33
	LSD	2.37	1.25	2.02	4.45	7.04
	Level of significance	**	**	**	**	**

Different potato genotypes and concentrations of GA3 influenced the length of roots per plantlet (Table 11). Maximum length of root (11.5 cm) was observed in Lal Pakri when Cultured in T2 (0.5 mg/l GA3) whereas minimum length of root (1cm) was observed in Jam Alu when cultured in T2 (0.5 mg/l GA3). No root formation was recorded in Shil Bilati when cultured in T1 (Fresh media), T2 (0.5 mg/l GA3), T3 (1.5 mg/l GA3) and in Kufri Shinduri when cultured in T1 (Fresh media) as well as in Jam Alu when cultured in T1 (Fresh media).

4.3.5. Number of leaves/ plantlet

Different potato genotypes and concentrations of GA3 significantly influenced the number of leaves/ plantlet. Data was recorded 21 DAI. The response of potato genotypes on number of leaves/ plantlet was found significant. Results presented in Table 9 showing that the maximum number of leaves/ plantlet (22.56) was recorded in Shil Pakri and minimum number of leaves/ plantlet (3.89) was recorded in Jam Alu. During the experiment Shil Bilati showed no response.

The effect of three different treatments was observed. Significant variations on number of leaves/ plantlet have been presented in Table 10. The maximum number of leaves/ plantlet (14.86) was recorded in T2 (0.5 mg/l GA3) and minimum number of leaves/ plantlet (8.45) was recorded in T1 (Fresh media).

Different potato genotypes and concentrations of GA3 influenced the number of leaves/ plantlet (Table 11). Maximum number of leaves/ plantlet (33.67) was observed in Shil Pakri when cultured in T2 (0.5 mg/l GA3) whereas minimum number of leaves/ plantlet (1.33) was observed in Jam Alu when cultured in T2 (0.5 mg/l GA3). Leaf was not formed in Shil Bilati when cultured in T1 (Fresh media), T2 (0.5 mg/l GA3), T3 (1.5 mg/l GA3) and in Kufri Shinduri when cultured in T1 (Fresh media).

From the above discussion it was found that Treatment T2 (0.5 mg/l GA3) showed promising performance in respect of maximum plant height (5.68 cm), number of branches/ plantlet (2.60), root length (8.74 cm) and number of leaves/ plantlet (14.86) and potato genotype Shil Pakri gave maximum

performance in respect of maximum plant height (8.96 cm), number of branches/ plantlet (2.78), number of roots/ plantlet (4.33) and number of leaves/ plantlet (22.56). Potato genotype Shil Pakri treated with T2 (0.5 mg/l GA3) gave maximum plant height (9.50 cm), number of branches/ plantlet (5) and number of leaves/ plantlet (33.67).

During the experiment Shil Bilati remained unresponsive to any of these hormonal treatments which were carried out in this experiment.

Plate 13: Subculture performance of Shil Bilati , Kufri Shinduri, Lal Pakri, Shil Pakri, Jam Alu and Pahari Pakri (from left to right) in T1 (MS media + 0 mg/l GA3)

Plate 14: Subculture performance of Shil Bilati , Kufri Shinduri, Lal Pakri, Shil Pakri, Jam Alu and Pahari Pakri (From left to right) in T2 (MS media + 0.5 mg/l GA3)

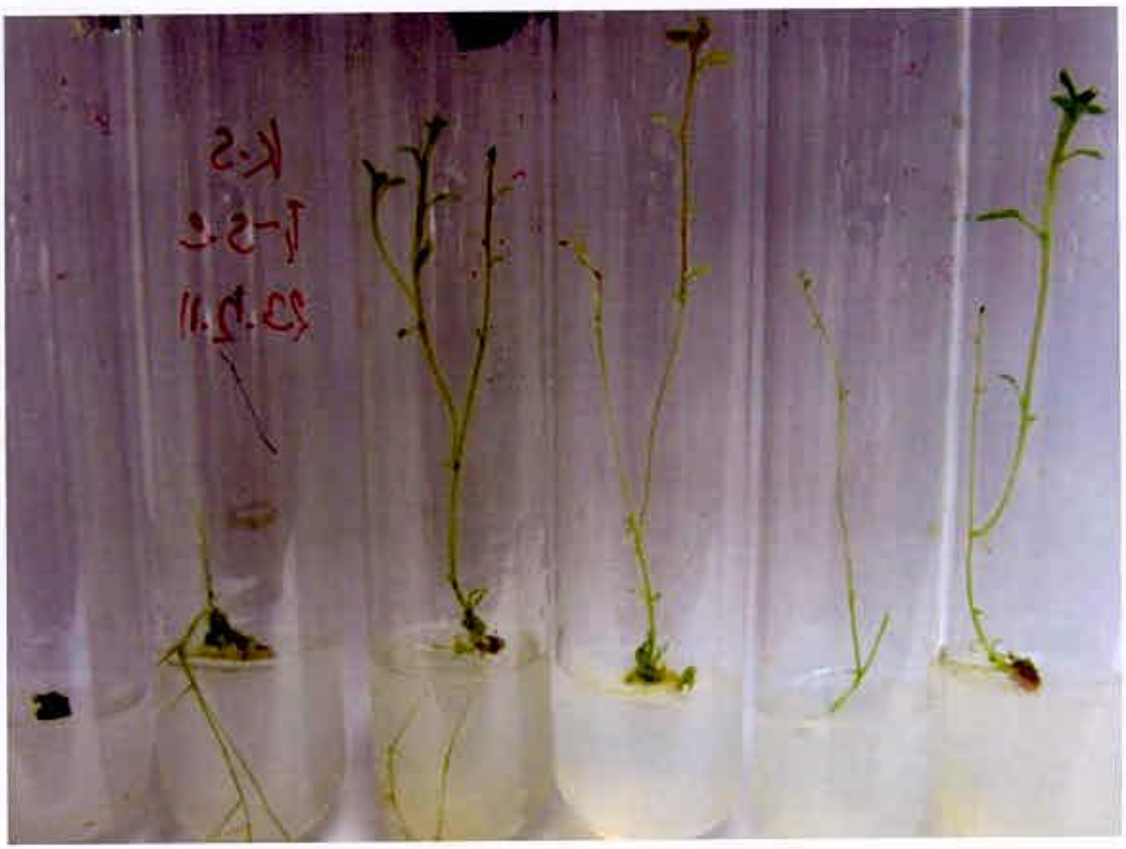

Plate 15 : Subculture performance of Shil Bilati , Kufri Shinduri, Lal Pakri, Shil Pakri, Jam Alu and Pahari Pakri (From left to right) in T3 (MS media + 1.5 mg/l GA3)

4.3.6. Subsequent multiplication of plantlets for the purpose of establishing plants in field condition

Plantlets derived from meristem culture need to be multiplied within short period of time for transferring plantlets in field conditions. For that purpose six potato genotypes were cultured in fresh MS along with T2 hormonal treatment (0.5mg/l GA3) for rapid multiplication of plantlet (Plate 16 & 17) using stem cutting techniques. This particular treatment was used because initially this treatment showed promising performance in rapid shoot elongation and growth of healthy plantlet *in vitro.* Explants used in shoot cutting were meristem culture derived *in vitro* shoots. Significant variations were observed in respect of plant height, number of branches/ plantlet, number of roots/ plantlet, root length and number of leaves/ plantlet (data presented in Table 12). Observations found are discussed under following heads.

4.3.7. Plant height

Significant variations were observed in respect of plantlet height among different potato genotypes when cultured in fresh MS media with 0.5 mg/l GA3. Maximum height (7.26 cm) was recorded in Shil Pakri and minimum height (1.92 cm) was recorded in Khufri Shindui (Figure 8).

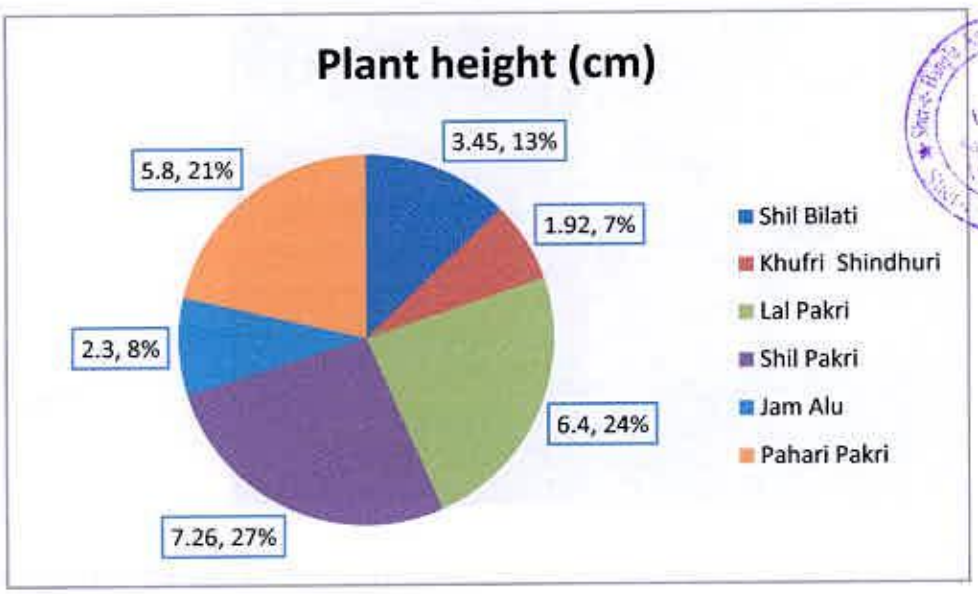

Figure 8: Effect of six potato genotypes on plant height

From above results and discussion it is obvious that among all six potato genotypes Shil Pakri gave maximum values in respect of plant height (7.26 cm), number of roots/ plantlet (5.49) and root length (7.14 cm) and number of leaves/ plantlet (11.07) whereas Jam Alu gave minimum values in respect of number of roots/ plantlet (2) and root length (4 cm) and number of leaves/ plantlet (4.42) but maximum number of branches/ plantlet (4). It is also found out that Kufri Shinduri gave minimum plantlet height (1.92 cm) and Shil Bilati gave minimum branches/ plantlet (0.75) among all genotypes.

4.4.11. *In vivo* hardening and establishment of plantlets in soil

Plantlets with well developed shoot root and leaves (preferably 15-21 days old) were removed from the culture vessels without damaging any roots. The culture media was washed away from the roots with running tap water. These plantlets were transferred to small trays with small pockets filled with well sterilized soil in growth chamber for 5 to 7 days. The soil was prepared as such that the proportion of soil was even with soil: sand: well decomposed cow dung (1:2:1). Immediately after transfer to trays those plantlets were sprayed with sterilized water and covered with transparent polythene to prevent desiccation. Survival rate of plantlets were 85 %, 90 %, 75 %, 90%, 75% and 75% in Shil Bilati, Kufri Shinduri, Lal Pakri, Shil Pakri , Jam Alu and Pahari Pakri respectively shown in Table 13.Then these trays were transferred to net house for another 10 days and proper care was taken for maximum growth and development of plantlets. After 10 days maximum plantlets showed vigorous growth and some less vigorous growth was also observed. After another 10 days plantlet were transferred to field condition in normal environment. The survival rate of plants in field condition were 0%, 60%, 40%, 80%,0% and 60% in Shil Bilati, Kufri Shinduri, Lal Pakri, Shil Pakri , Jam Alu and Pahari Pakri respectively as shown in Table 14.

Table 13: Survival rate of *in vitro* regenerated plantlets in growth chamber

Potato genotype	Number of plants transplanted in tray	Number of plants survived	Survival percentage (%)
Shil Bilati	20	17	85
Kufri Shinduri	20	18	90
Lal Pakri	20	15	75
Shil Pakri	20	18	90
Jam Alu	20	15	75
Pahari Pakri	20	15	75

Table 14: Survival rate of transplanted plantlets in field condition

Potato genotype	Number of plants transplanted	Number of plants survived	Survival percentage (%)
Shil Bilati	15	0	0
Kufri Shinduri	15	9	60
Lal Pakri	15	6	40
Shil Pakri	15	12	80
Jam Alu	15	0	0
Pahari Pakri	15	9	60

4.4. ELISA test:

Successfully *in vitro* grown plantlet samples from each genotypes were send to TCRC, BARI Laboratory for conducting a serological identification of plantlets against five viruses PLRV, PVY, PVX, PVS and PVM through double antibody sandwitch enzyme-linked immunosorbent assay (DAS-ELISA) protocol (BARI, 2012). The result of the ELISA test is given below;

Table 15: ELISA report

Sample No.	Antiserum used				
	PLRV	PVY	PVS	PVX	PVM
Shil Bilati	-	++	-	++	-
Kufri Shinduri	-	-	-	-	-
Lal Pakri	-	-	-	-	-
Shil Pakri	-	-	-	-	-
Jam Alu	-	+	++	+++	-
Pahari Pakri	-	-	-	-	-

Note: '+' marks means virus is present in this sample.
'-' marks means virus is absent in this sample.

Plate 18: Plantlets transferred to sterilized soil containing tray

Plate 19: A. Plant hardening in net house, B and C. Healthy plants are growing in normal field environment

Chapter 5

Summary And Conclusion

CHAPTER 5

SUMMARY AND CONCLUSION

The experiment entitled "Meristem culture and *in vitro* plant regeneration of some potato (*Solanum tuberosum* L.) genotypes" was conducted at the tissue culture laboratory of Dept. of Biotechnology, Sher-E-Bangla Agricultural University, Sher-E-Bangal Nagar, Dhaka, during the period from March 2010 to October 2011. Three different sets of experiment were conducted and protocols were developed. The first experiment was conducted to investigate sprouting abilities of six potato genotypes
(Shil Bilati, Kufri Shinduri, Lal Pakri, Shil Pakri , Jam Alu and Pahari Pakri) using potato tubers as explants. The second experiment was carried out to study the callus induction abilities and subsequent plant regeneration using meristems of sprout tips as explants. The last experiment was conducted to study *in vitro* plant growth and establishment of meristem culture derived single shoots and *in vivo* establishment of these genotypes. The experiment was designed following Completely Randomized Design (CRD) with ten, five and three replications.

The first experiment was started with primary materials grown in different parts of Joypur, Joypurhat and Rangpur and was collected from Bangladesh Agricultural Development Corporation (BADC). Six potato genotypes of similar size, shapes and weight were selected and cleaned for conducting the experiment. Four levels of GA3 treatments (100, 200, 300 and 400 ppm) were used to spray regularly two times a day on selected and cleaned potatoes with a view to obtain highest sprouts from relatively short period of time. Among four treatments 400ppm GA3 showed most effective effect on sprout length, number of sprout per potato, number of sprout per eye in minimum days compared to other treatments in all six genotypes. The best treatment (400ppm) was applied to initiate subsequent sprouting to obtain large numbers of sprouts for collecting meristems from sprout tips for conducting experiment 2 successfully.

In the next experiment meristem was excised from potato sprouts and used as explants. Seven levels of treatments (T0, T1, T2, T3, T4, T5 and T6) was used to study effects on six potato genotypes regarding callus initiation, callus size and single shoot regeneration. Extensive investigation was done to find out the best treatment for callus induction from meristem. Their survival percentage also investigated after inoculation of meristem in MS containing different hormonal treatments. Maximum survival percentage were observed in all genotypes when cultured in T0 (fresh MS media without any hormone combinations) where as minimum survival percentage (40%, 60%, 70% and 80%) was recorded in T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/l KIN) and T4 (0.5mg/l GA3+1.0mg/l 2,4-D+1.5mg/lKIN) in different potato genotypes. The lowest survival percentage (40%) obtained by genotype Pahari Pakri in T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/lKIN). The texture of Kufri Shinduri and Jam alu was non friable and other four genotype developed friable callus. Color of different genotypes developed four different colors. The colors of callus were whitish green, yellowish green, greenish and reddish (Table 5).

MS media supplemented with T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN) initiated callus in minimum days (3.33days). T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN) produced callus and took maximum days (9.17 days) to initiate callus. Shil Pakri required maximum days (7.68 days) to initiate callus and Shil Bilati initiated callus in very short period of time (3.66 days). Shil Pakri cultured in T5 (0.5 mg/l GA3 +1 mg/l 2, 4-D+ 0.5mg/l KIN) initiated callus in 15 days and Shil Bilati cultured in T4 (0.5 mg/l GA3 +1 mg/l 2, 4-D+ 1.5mg/l KIN) initiated callus in one days. No callus was developed in MS media supplemented without hormones.

Maximum callus size was recorded (0.66 cm) in Kufri Shinduri whereas minimum callus size was recorded (0.29 cm) in Shil Bilati. Best treatment for producing large size callus (0.54 cm) was found to be T4 (0.5mg/l GA3 + 1 mg/l 2, 4-D + 1.5mg/l KIN) and minimum callus size (0.31 cm) was observed in T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/l KIN). Maximum size of callus was observed (0.82 cm) when meristem of Kufri Shinduri inoculated in T4 (0.5 mg/l GA3 +1.0 mg/l 2, 4-D+ 1.5 mg/l KIN) whereas minimum size of callus was

recorded (0.17 cm) when meristem of Shil Bilati was inoculated in T1 (0.5 mg/l GA3 +0.5 mg/l 2, 4-D+ 0.5 mg/l KIN).

Hormonal treatment T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN) initiated single shoot from callus only in 5.13 days which was minimum days required for single shoot initiation. Highest single shoot length (2.41 cm) was produced by Shil Pakri 30 DAI and shortest shoot length (0.27 cm) was recorded in Shil Bilati 30 DAI. From the observations during conducting experiment and extracted data treatment T3 was found most effective hormonal treatments to initiate single shoot and regeneration. Among all genotypes Shil Pakri was the best genotype and produced maximum shoot length (5.88 cm).

In third experiment three levels of GA3 treatments were used to study their effect on six potato genotypes to study healthy and strong plantlet development. Results revealed that treatment T2 (0.5 mg/l GA3) gives promising performance in respect of maximum plant height (5.68 cm), number of branches/ plantlet (2.60), root length (8.74 cm) and number of leaf (14.86) and variety Shil Pakri gave maximum performance in respect of maximum plant height (8.96 cm), number of branches/ plantlet (2.78), number of roots/ plantlet (4.33) and number of leaves/ plantlet (22.56). Shil Pakri treated with T2 (0.5 mg/l GA3) gave maximum plant height (9.50 cm), number of branches/ plantlet (5) and number of leaves (33.67). During the experiment Shil Bilati remained unresponsive to any of these hormonal treatments which were carried out in this experiment.

The best treatment T2 (0.5mg/l GA3) found was used to further multiply plantlet by shoot cutting. Data recorded from shoot cutting showed that Shil Pakri gave maximum values in respect of plant height (7.26 cm), number of roots/ plantlet (5.49) and root length (7.14 cm) and number of leaves/ plantlet (11.07) whereas Jam Alu gave minimum values in respect of number of roots/ plantlet (2) and root length (4 cm) and number of leaves/ plantlet (4.42) but maximum number of branches/ plantlet (4). It is also found out that Kufri Shinduri gave minimum plantlet height (1.92 cm) and Shil Bilati gave minimum number of branches/ plantlet (0.75) among potato genotypes.

For acclimatization plantlets were transferred from culture media to tray containing sterile soil. Survaival rate varied from 90 to 75 %. Highest survival rate was recorded in Kufri Shinduri and Shil Pakri whereas lowest in Lal Pakri, Jam Alu and Pahari Pakri. When plantlets grown in trays were transferred to field condition where survival rate greatly varied from 0 to 80%. Potato genotypes which did not survived in field condition are Shil Bilati and Jam Alu whereas maximum survival rate was recorded in Shil Pakri.

The result of present investigation indicated that 400ppm GA3 can be sprayed to get sprouts of desirable amount and Shil Pakri can be successfully treated with T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN) to generate callus and single shoot regeneration. Subsequent culture of single shoot can be done in MS media containing 0.5 gm/l GA3 to get healthy plantlet for rapid shoot multiplication. Among all potato genotypes Shil Pakri showed better overall performance in plant establishment from meristem culture to establishment of plants in field conditions. Plantlets sent to TCRC, BARI for ELISA test confirmed that two Shil Bilati and Jam Alu was infected with viruses (PVX, PVS and PVY) after meristem culture but other genotypes gave virus negative results (Table 15).

More research should be done to study and find out effective hormonal treatments suitable for Shil Bilati which showed poor performance during this investigation. Effective light intensity needed by these potato genotypes to grow in *in vitro* conditions should be studied in future.

Chapter 6
References

CHAPTER-6

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Chapter 7
Appendices

CHAPTER-7

APPENDICES

Appendix - I: Composition of MS medium (Murashige and Skoog, 1962)

Components	Concentrations (mg/L)
Macronutrients / Major salts	
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
Micronutrients / Minor salts	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.4H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
Iron Sources	
$FeSO_4.7H_2O$	27.80
Na_2EDTA	37.30
Vitamin and Organic Nutrients	
Thiamine (HCl)	0.1
Niacine	0.5
Glycine	2.0
Pyrodoxine (HCl)	0.5
Glycine	2.00
Myo inositol	100
Sucrose	3000.00
Agar	8000.00
pH adjusted to 5.8 before autoclaving	

Appendix -II: ANOVA table for effect of GA3 treatments on sprouting abilities of six potato genotypes

Source	Degrees of Freedom	Days required to initiate sprouting	Number of sprouts/ potato	Maximum Sprout length (cm)	Number of sprouts/ eye
Factor A	5	192.825	567.2	2.74	31.925
Factor B	3	212.458	156.167	9.401	3.125
AB	15	44.958**	18.067**	0.838**	0.725**
Error	48	0.0001	0.542	0.056	0.417

** = Significant at 1% level

* = Significant at 5% level

NS= Non Significant

Appendix -III: ANOVA table for the effect of six potato genotypes on days to callus initiation and proliferation

Source	Degrees of freedom	Days to callus initiation	Size of callus		
			length (cm)	Breadth (cm)	Callus size (cm)
Factor A	5	61.133**	0.112**	1.479**	0.594**
Factor B	5	120.933**	0.059**	0.579**	0.239**
AB	25	23.867**	0.044**	0.165**	0.059**
Error	144	2.325	0.005	0.027	0.01

** = Significant at 1% level

* = Significant at 5% level

NS= Non Significant

Appendix -IV: ANOVA table for the effect of different hormonal treatments and potato genotypes on regeneration of single shoot/ callus

Source	Degrees of freedom		Shoot length (cm)			
		Days to shoot initiation	14 DAI	21 DAI	30 DAI	Days to root initiation
Factor A	5	2.28**	173.102**	1.067**	431.947**	64.773^{NS}
Factor B	5	1.013**	50.262**	5.547**	89.075**	60.76^{NS}
AB	25	0.349**	21.364**	1.373**	64.405**	16.829^{NS}
Error	144	0.053	5.986	0.267	9.128	2.436

** = Significant at 1% level

* = Significant at 5% level

NS= Non Significant

Appendix -V: ANOVA table for the effect of 21 days old subculture performance and different GA3 hormonal treatments for rapid multiplication of single shoot obtained from meristem culture

Source	Degrees of freedom	Plant height (cm)	Number of branches/ plantlet	Number of roots/ plantlet	Root length (cm)	Number of leaves/ plantlet
Factor A	5	173.102**	1.067**	431.947**	64.773**	151.609**
Factor B	5	50.262**	5.547**	89.075**	60.76**	80.102**
AB	25	21.364**	1.373**	64.405**	16.829**	21.319**
Error	144	5.986	0.267	9.128	2.436	6.302

** = Significant at 1% level

* = Significant at 5% level

NS= Non Significant

Appendix -VI: ANOVA table for the effect of different potato genotypes on subculture performance

Source	Degrees of freedom	Plant height (cm)	Number of branches/plantlet	Number of leaves/ plantlet	Number of roots/ plantlet	Root length (cm)
Factor A	5	85.81**	7.496**	26.019**	115.608**	611.663**
Factor B	2	7.97**	17.241**	1.13**	32.594**	192.241**
AB	10	4.593**	2.374**	4.307**	33.141**	66.841**
Error	36	2.051	0.574	1.481	7.217	18.074

** = Significant at 1% level

* = Significant at 5% level

NS= Non Significant