VIRUS FREE PLANTLET PRODUCTION OF POTATO (*Solanum tuberosum* L.) THROUGH MERISTEM CULTURE

BY

MD. HARUN- OR- RASHID

DEPARTMENT OF PLANT PATHOLOGY
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA-1207

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BY

MD. HARUN- OR- RASHID
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Approved By:

Dr. Md. Belal Hossain Associate Professor Department of Plant Pathology Sher-e-Bangla Agricultural University Sher-e-Bangla Nagar, Dhaka-1207 **Supervisor**	**Dr. Fatema Begum** Associate Professor Department ofPlant Pathology Sher-e-Bangla Agricultural University Sher-e-Bangla Nagar, Dhaka-1207 **Co-Supervisor**

Dr. Md. Belal Hossain
Chairman
Examination Committee

Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207

PABX: +880244814045
Fax: +88028155800
E-mail: dr.mbhossain@sau.edu.bd
Web-site: www.sau.edu.bd

CERTIFICATE

*This is to certify that the thesis entitled, "**VIRUS FREE PLANTLET PRODUCTION OF POTATO (Solanum tuberosum L) THROUGH MERISTEM CULTURE**" submitted to the Department Of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE IN PLANT PATHOLOGY**, embodies the result of a piece of bona fide research work carried out by* **MD. HARUN- OR- RASHID** *bearing **Registration No. 09-03373** under my direct supervision and guidance. No part of this thesis has been submitted for any other degree in any other institutions.*

I further certify that any help or sources of information received during the course of this investigation have been duly acknowledged.

Dated: 01.12.2016

Place: Dhaka, Bangladesh

Dr. Md. Belal Hossain
Associate Professor
Department of Plant Pathology
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar,Dhaka-1207
Supervisor

Dedicated To

My

Beloved Parents

LIST OF ABBREVIATION AND SYMBOLS

Abbreviation	Full words
^{0}C	Degree Celsius
%	Percentage
1N	1 Normal
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
ha	Hectare
ha^{-1}	Per Hectare
h.	Hours

LIST OF ABBREVIATION AND SYMBOLS (Cont'd)

Abbreviation	Full words
$HgCl_2$	Mercuric chloride
i.e.	ed est (means that is)
KIN	Kinetin
1M	Indole-3-Acetic Acid
NM	α-napthalene acetic acid
NaCI	Sodium Chloride
Int.	International
J.	Journal
Mg	Milligram (s)
mg/I	Milligram per liter
MI	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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Author

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ABSTRACT

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The experiment was undertaken with a view to establish a protocol for *in vitro* culture and plant regeneration using potato meristem as explant collected from potato sprouts of six popular potato varieties Diamant, Cardinal, L. Rosetta, Granola, Asterix and BARI ALU 29. The experiment was setup at Tissue culture laboratory of Debiganj farm, Panchagarh. from March, 2015 to February, 2016. In the present study four levels of GA3 (100, 200, 300 and 400 ppm) were used to assess the influence of GA3 on sprouting abilities of six popular potato verities. The maximum sprouting efficiency was observed in 400 ppm GA3 treatment. Seven levels of GA3, 2, 4-D and Kinetin were used along with fresh MS media to inoculate meristems of potato sprouts. Maximum size of callus (0.82 cm) was observed in Cardinal inoculated in T3 (0.5 mg/l GA3 +1.0 mg/l 2, 4-D+ 1.5 mg/l KIN). Granola meristem inoculated in hormonal treatment T2 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.5mg/l KIN) showed the best results regarding minimum days required to shoot initiation (5.13 days) and the single shoot length (2.41 cm). Three levels of GA3 treatments (T1- fresh MS, T2- MS+0.5mg/l GA3 and T3- MS+1.5mg/l) were used to study the subsequent shoot elongation and plant regeneration of single shoot produced from meristem culture. On DAS- ELISA test all genotypes showed negative result against virus. Among all varieties Diamant was the least responsive whereas Granola showed overall better performance from meristem culture to establishment of plants in field conditions.

CHAPTER 1

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the important tuber food crops of the world. It is used as a staple food in many countries of the world it is but mainly used as a vegetable in Bangladesh (Hussain, 1995). The potato ranks 4th among the important food crops in the world including, wheat, rice and corn. Potato is very nutritious tuber vegetable. Potato is the rich source of starch, vitamin C, B and minerals. It also contains a good amount of essential amino acids like leucine, tryptophan and isoleucine. Potato also contains a variety of phytonutrients that have antioxidant activity.

Potato as vegetables alone contributes about 64% of total annual vegetable production in Bangladesh (BBS, 2011). The varieties of potato introduced to Bangladesh after 1960. However, with the introduction of modern potato varieties from Holland during the early sixties, the area and production of potato in Bangladesh began to increase rapidly and all of the new areas were covered by the modern varieties. In the year 2011- 2012, potato was grown in an area of 43 millions of hectares and the production was 82 millions of metric tons (BBS, 2012). Traditional or 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 but these varieties are still widely grown due to their popularity among growers for longer self life, good keeping quality in storage, low cost of production, reasonably good yields with low inputs, tolerant to stress conditions, high market demand and for giving desired yield even in pathogenic attack and insect infestation. Among those desired varieties Diamant and Cardinal are preferred by the consumers due to better taste (liangantileke *et al.*, 1999-2000).

Potato is cultivated twenty three major growing areas in Bangladesh. In comparison to other agricultural crops, the seed cost of potato cultivation is much higher. Bangladesh Agricultural Development Corporation (BADC) reported that the seed cost of potato is about 30 - 40% of total production cost (Anon *et al.*, 2005). Bangladesh imported high yielding foreign potato varieties at the cost of around US $ 150.00 per quintal which engross large amount of foreign currency every year (Ahloowalia *et al*, 2010). Potato varieties are susceptible to viral diseases which may cause up to 75% yield losses. Some viruses can decrease the yield by 40% singly and in combination with other viruses, the loss may raise 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 % yield by using virus and disease free quality seeds (Karim *et al.,* 2011). These viral diseases are tuber born. Virus contaminations 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 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). High yielding foreign potato varieties significantly increased the yield of potato crop in our country but at the same time results in new viral problems like *PLRV, PVY* and *PVX* which have been reported in Bangladesh and causes 10-90% yield losses.

Being one of the important cash crops, potato yields higher net return although it requires more labor input than any other crops. But commercial potato production is a complex and highly specialized commercial enterprise that demands a high degree of technical skills and practical experience on the plant .In many developing countries including Bangladesh, yields of root and tuber crops are significantly reduced to their potential due to seed-borne diseases and pests. In vegetative propagated potato crops, once the plant systematically infected with a viral disease, the pathogen is transmit from one generation to next. There are approximately 23 virus and virus like organisms that cause disease in potato. Khan (1981) reported that a single plant of potato may be infected with four to five viruses. The presence of viral disease is an important reason attributed to low yield of potato varieties in our country (Ahmed, 2001; Siddique and Hussain, 2012).

Currently, no reports are available about high yielding commercial varieties or advance potato lines in Bangladesh that shown durable resistance against viruses (Karim *et al.,* 2010). Most viruses can effectively be determined by ELISA tests, but the serological methods can be unreliable for the detection all potato viruses like *PLRV,* because this virus often occurs at low concentration in plant tissue and virions are weakly immunogenic (Abdul *et al*., 1987). But reports said that meristem culture technique is effective for elimination of virus (Morel and Martin, 1955). Production of virus

free plantlet through meristem culture technique also has been reported in many crops (Bhojwani and Razdan, 1983). *In vitro* meristem culture has appeared a new venture in obliging virus free potato tuber seeds. Zhang (1995) reported 40% yield increase in potato using virus free tuber seeds. Similar techniques can be applied for potato production in agriculture of Bangladesh. The prime goal of the proposed study is to develop reproducible protocol for producing of virus free potato seed tubers applicable for Bangladesh. The success of this experiment will led to additional financial support from respective government to government and non-government entrepreneurs for producing diseases free potato tuber seeds in future.

OBJECTIVES:

In view of the above facts, the present research work was carried out to achieve the following objectives:

-To establish virus free regeneration protocol of some popular potato genotypes in *in vitro*.

-To find out the best hormonal combination for meristem culture in the laboratory.

-To study rapid multiplication of virus free potato plantlet through micro-propagation.

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 technique (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). 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).

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2.3. Concept of meristem culture

The apical meristem together with one to three young leaf primordia, measuring 0.1-0.Smm, 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 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 cu Itu re. 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 kinetinlitre alone to the medium was ineffective to increase survival and percent regeneration. MS medium supplemented with 0.2 mg kinetin + 10 mg GA3 1itre 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 one 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 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 primordial of two sweet potato cultivars, Yushu 4 and Yushu 12, were cultured for 20 days on MS + BA (0.5 mg/litre) + 1M (0.1 mg/litre) + GA3 (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° 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/I Kinetine and 0.1 mg/I NAA) and MSKN3 (1 mg/I Kinetine and 0.1 mg/I 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).

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 (9cm) 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 Ix 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>SA>GA3. The optimum combination was reported 0.25 mg NAA + 1.5 mg SA + 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, in a callus induction in potato. In addition, the effects of cultivar and explant were evaluated on callus.

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, Santé, Concord, Ajax and Oiamant) 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.

2.7. 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).

2.8. *In vitro* plant regeneration and multiplication:

[illegible] were done in potato. These methods allow rapid

Malia *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 0.01 mg/l IAA, 0.20 mg/l GA3, 44.0 mg/l CaCI2 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 was 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/I sucrose and 6 g/l gar. 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 was concluded that when

heterogeneous explants were used, the initial characteristics of the explants could 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 gibberellicacid, 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, zip (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

3.1. Experimental site

The research experiment was carried out at Tissue Culture Laboratory of Debiganj Agricultural farm, BARI, Panchagarh.

3.2. Experimental duration

The experiment was conducted from March, 2015 to February, 2016 to obtain *in vitro* regeneration of meristem cultured plant materials of six potato varieties.

3.3. Variety Selection and Collection of Seed tubers

Popular varieties of potato grown in major potato growing areas of Bangladesh were selected for the study, and seed tubers were collected from Bangladesh Agricultural Development Corporation (BADC), Gazipur.

3. 4. Primary plant materials

The experimental materials of selected six potato varieties are listed below-

- Diamant
- Cardinal
- Lady Rosetta
- Granola
- Asterix
- BARI ALU -29

3.5. General experimental protocol

Primary plant materials of six potato varieties were treated with GA3 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 medium with hormones suitable for rapid multiplication of plantlets was done.

3.6. 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. Before GA3 treatment potato varieties were gently washed with liquid detergent (Twin-20) 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 followed by placed on blotter paper 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). 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 400 ppm 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 sprouted potato. GA3 concentration of 400 ppm 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

3.7. 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. 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.

3.8. Preparation of stock solutions

MS medium was prepared by combination of stock solutions with different minerals and hormones required for plant growth and 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 were dissolved in double distilled water or highly purified de-ionized water. Each chemical were added according to the list of ingredients presented in Appendix-I

3.8.1. Preparation of stock solution of macro nutrients (stock I)

Stock solution of macro nutrients or stock I was prepared with 10 times (10X) of the final strength of the medium in 1000 ml of distilled water. Salts are weighted accurately and dissolved completely ten times the weight of the salts red for one liter 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 ± 1^0 C for future use.

3.8.2. Preparation stock solution of micronutrients (stock II)

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 l. The stock solution was filtered as same manner as described earlier and labelled and stored in a refrigerator at 4 ± 1^0 C.

3.8.3. Preparation stock solution of Iron-EDTA (stock III)

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. FeS04 and Na-EDTA were dissolved in 750ml 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 aluminum foil. The Prepared bottle was stored in refrigerator a 4 ± 1°C.

3.8.4. Stock Solution for growth regulators

Stock solution I, II and III are required to prepare MS medium. There were 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, and 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 ± 1^0 C. Newly prepared growth regulators could be used maximum for two months. After the expired date new growth hormones were prepared for experimental use.

3.9. Preparation of other stock solutions

3.9.1. 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.

3.9.2. 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.

3.10. 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.

3.10.1. Preparation of MS media

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

- ✓ 100ml of stock I, 10ml of stock II, and 10ml of stock III 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 the 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 HCI were used as per requirement.
- ✓ 8 gm Agar was added to the mixture and heated in a micro wave oven at 100 ^{o}C slowly until the agar dissolves completely.
- ✓ The MS medium is now ready to sterilize and after sterilization it will be ready to use.

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

3.11. Sterilization steps

3.11.1. Sterilization of culture media

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

3.11.2. 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 aluminum foil and autoclaved at 15 psi pressure at 121 $^{\circ}$C for 30 minutes.

3.11.3. 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. Highest precautions were 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.

3.11.4. 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.

Figure 1: Sterilized glass-wares in sterile laminar air flow cabinet.

3.12. 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^{O}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.

3.13. Protocol of meristem culture

The general meristem culture procedure was carried out as follows:

3.13.1. Explants

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

3.13.2. Surface sterilization and meristem culture

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 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 % $HgCI_2$ 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.

Figure 2: Primary plant materials. A. Diamant, B. L. Rosetta, C. Granola, D. Asterix, E. BARI ALU 29, F. Cardinal .

3.13.3. 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. 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.

3.13.4. 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.

3.13.5. Continuous sub culture of shoot cuttings

After getting the successful plantlets from meristem continuous sub culturing was done to get vigorous plantlets using 2 cm long shoot cutting. After doing 3-4 subs culturing all the plants showed vigorous growth at high light intensity.

3.13.6. Treatments

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

3.14. List of experiments and Data collection methods

3.14.1. 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 (100 ppm, 200 ppm, 300 ppm and 400 ppm

Parameters: Data was collected under following parameters;

3.14.1.1. Days required initiating 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 as 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.

3.14.1.2. 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.

3.14.1.3. Maximum sprout length (cm): Mximum sprout length in centimeters was taken and recorded from 30 days old potatoes treated with different concentrations of GA3.

3.14.1.4. 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.

3.14.2. Experiment 2: Effect of 2, 4-0, KIN and GA3 on meristem culture, callus induction and shoot regeneration of six potato genotypes.

Design: CRD

Replication: 3

Explant: Meristem / shoot tip from potato sprouts, Number

of explants inoculated per replication: 1

Treatment details

Treatment s	Components with composition of 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 find out the best combination for

meristem culture. In these 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.

3.14.2.1. 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

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

3.14.2.2. Days to callus initiation

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

3.14.2.3. 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:

$$\text{Callus size} = \frac{\text{Length+ breadth}}{2} \times 100$$

3.14.2.4. 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 as days to shoot initiation. When single shoot was visible then it was recorded as days required shooting initiation.

3.14.2.5. Shoot length/plantlet

Shoot length of cultured explants was recorded at 14, 21 and 30 Day After Incubation (DAI).

3.14.2.6. Days to root initiation

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

3.15. ELISA test (Experiment 3)

Primary plant materials were collected from viral disease infected potato fields during cultivation in winter season, 2014-2015. Virus infected plants noticed in field conditions were recorded and photos were taken. 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 obt culture were virus free.

Figure 3: ELISA machine

3.16. Acclimatization

Well-developed plantlet with developed root was removed from the culture vessels carefully without damaging the roots. Culture medium was washed away from the roots with running tap water. Then 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 decomposed cow dung and ashes (1:1 :1). This tray was kept in the hardening room for 7 days.

3.17. Transfer of plantlets to soil

After acclimatization the plantlets were transferred to net house, where proper care was taken for growth and development. After 20 dayplantlet showed maximum growth and was transferred to the normal field condition.

3.18. Experimental design and statistical data analysis

All the *in vitro* experiments such as meristem culture, callus initiation, plant regeneration, and subculture were done under controlled laboratory condition, using completely randomized design (CRD). The collected data on different parameters were analyzed using an 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

For regeneration of potato plantlets from meristem culture was studied to develop reproduction protocol with a view to supply virus and disease free planting materials in large quantities for large scale cultivation. *In vitro* regeneration of six potato pgenotypes using different combinations of GA3, 2, 4-D and KIN on meristem culture, shoot multiplication were explored. The result obtained from this experiment have been presented and discussed in this chapter.

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

In this present experiment meristem was collected from potato sprouts. Four levels of solutions concentrated with GA3 (100, 200, 300 and 400 ppm) was sprayed to six potato genotypes. Results have been presented in Table 1. Various treatments showed significant influence over days required to initiate sprouting, number of sprouts/ potato, maximum sprout length and number of sprouts/eye.

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

4.1.1.1. Days required initiating sprouting

Diamant, Cardinal, L. Rosetta, Granola, Asterix and BARI ALU 29 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 same varieties were 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, Asterix responded slowly (29 days required to initiate sprouting) in 100 ppm GA3 application but responded quickly to initiate sprouting (only within 3 days) in 400 ppm GA3 application. BARI ALU 29 took the shortest possible time (1 day) to initiate sprouting when it was treated with 400 ppm GA3 solutions. From this study it was found that, Asterix was 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. BARI ALU 29 was found to be less dormancy and easily

responding to 100, 200, 300 and 400 ppm GA3 treatments (4, 2, 2 and 1 days required to initiate sprouting, respectively) (Table 1).

4.1.1.2. Number of sprout /potato

A significant increase in number of sprouts/potato was observed with increasing concentration of GA3 application. Diamant, Cardinal, L. Rosetta, Granola, Asterix and BARI ALU 29 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 was recorded in Cardinal (27) and Granola (18) under 400 ppm GA3 applications. Minimum number of sprouts was recorded in L. Rosetta under 100 ppm GA3 applications. The above observations illustrated that increasing level of GA3 treatment enhances sprouting intensity quickly and effectively (Table 1).

4.1.1.3 Maximum sprout length

Maximum sprout length was recorded in Lady Rosetta and BARI ALU-29 (3.50 cm) under 400 ppm GA3 application and minimum length of sprout was recorded in Lady Rosetta (0.20 cm) under 100 ppm GA3 application (Table 1). Almost all potato genotypes showed significant variation in response to four hundred 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 four hundred ppm desirable in this experiment to get maximum number of sprouts comparatively in short period of time. 400 ppm GA3 treatment was selected as the best treatment and used to get maximum sprouts for the collection of meristematic shoot tip in shortest possible time. The present results are similar with the report of Jing, 2014.

4.1.1.4. Number of Sprouts/ eye

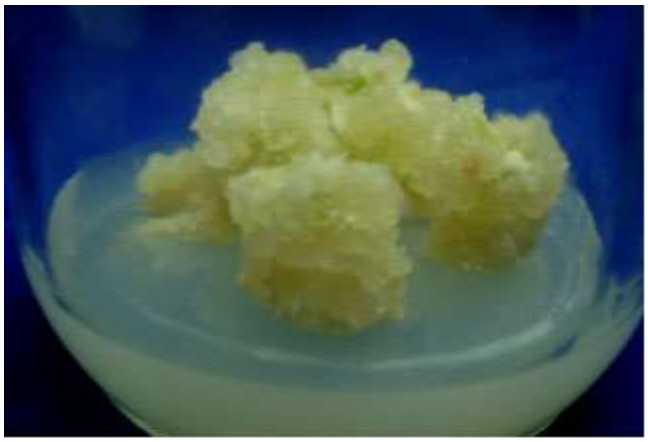
The rate of sprouts/potato observed in this experiment due to the application of concentration of GA3 was found more or less same with other parameter. Among six varieties Granola showed the highest number (7) of sprouts/ eye in response to 400 ppm. However Asterix showed the lowest number of sprouts/ eye and it were just 2 in response to 400 ppm GA3 treatment (Table 1).

Table 1: Effect of GA3 treatment on sprouting abilities of six potato varieties

Potato genotype	GA3 treatment (ppm)	Days required to initiate sprouting	Number of Sprouts/ potato	Maximum Sprout length (cm)	Number of Sprouts/ eye
Diamant	100	5	5	1	3
	200	5	7	1.3	3
	300	5	9	1.3	3
	400	3	10	1.3	3
Cardinal	100	8	13	1	4
	200	5	19	1.2	4
	300	5	25	1.5	4
	400	4	27	2.2	4
L. Rosetta	100	29	2	0.2	1
	200	14	3	1.5	1
	300	9	3	2	1
	400	3	5	3.5	3
Granola	100	4	6	2.3	5
	200	2	7	2.3	5
	300	2	7	2.3	5
	400	1	18	3.1	7
Asterix	100	8	1	0.5	1
	200	5	2	2	1
	300	4	2	2.5	1
	400	2	5	3.1	2
BARI ALU 29	100	8	3	1	4
	200	5	3	2	4
	300	3	5	2.5	4
	400	2	7	3.5	4
	LSD	0.07	1.21	0.39	1.06
	Level of significance	**	**	**	**

Complete protocol of plantlets production

A

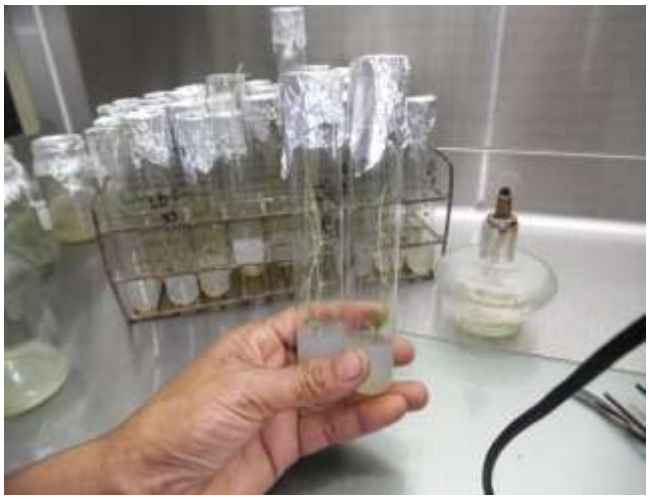


B

C

D

E



F

Figure 4: A. Sprouted potato, B. Callus Formation, C. Culture media, D. 21 days old meristem observation, E. Cutting of 21 days old meristem, F. 30 days old meristem observation.

4.2.1. Days to callus initiation:

Meristems of six potato varieties were isolated from freshly grown sprouts and cultured in different combinations of GA3, 2, 4-D and kinetin. Results on effects of different varieties are presented in Table 2 and 3.

The Effect of different hormonal combinations on days to callus initiation and proliferation has been presented in Table 2.The maximum 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 the 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.

The potato genotypes and different levels of hormonal treatments showed significant interaction in relation to the required days to callus initiation (Table 3). The maximum days (15 days) required for callus initiation was observed in Granola cultured in T5 (0.5mg/l GA3 +1 mg/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). The minimum day (1 day) required for callus initiation was observed in Diamant when cultured in T4 (0.5 mg/l GA3 +1 mg/l 2, 4-D+ 1.5mg/l KIN).

4.2.2. Size of callus

A significant difference in size of callus was observed among selected potato genotypes (Table 2). Maximum callus size was recorded in Cardinal (0.66 cm) whereas minimum callus size was recorded in Diamant(0.29 cm). Hormonal treatments T4 (0.5 mg/lGA3 +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) and T6 (0.5 mg/l GA3 +1.0 mg/l 2, 4-D+ 1.0 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 3). Maximum size of callus was observed (0.82 cm) when meristem of Cardinal was inoculated in T4 (0.5 mg/l GA3 +1.0 mg/l 2, 4-D+ 1.5 mg/l KIN) whereas the minimum size of callus was recorded (0.17 cm) when meristem of Diamant 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 2: Effect of different hormonal treatments on days to callus initiation and proliferation

Treatment	Days to callus initiation	Callus Size
To	-	-
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
LSD	0.78	0.05
Level of significance	**	**

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

Potato genotype	Treatment	Days to callus initiation	Callus Size (cm)
Diamant	T0	-	-
Cardinal	T0	-	-
L. Rosetta	T0	-	-
Granola	T0	-	-
Asterix	T0	-	-
BARI ALU 29	T0	-	-
Diamant	T1	9.00	0.17
Cardinal	T1	7.00	0.39
L. Rosetta	T1	8.00	0.34
Granola	T1	5.00	0.24
Asterix	T1	6.00	0.30
BARI ALU 29	T1	5.00	0.28
Diamant	T2	4.00	0.31
Cardinal	T2	3.00	0.61
L. Rosetta	T2	5.00	0.42
Ganola	T2	3.00	0.23
Asterix	T2	5.00	0.28
BARI ALU 29	T2	6.00	0.28
Diamant	T3	2.00	0.27
Cardinal	T3	5.00	0.73
L. Rosetta	T3	3.00	0.50

Table 3 Continued……...

Granola	T3	2.00	0.58
Asterixc	T3	3.00	0.71
BARI ALU 29	T3	5.00	0.37
Diamant	T4	1.00	0.41
Cardinal	T4	5.00	0.82
L. Rosetta	T4	5.00	0.28
Granola	T4	9.00	0.38
Asterix	T4	10.00	0.40
BARI ALU 29	T4	9.00	0.35
Diamant	T5	3.00	0.23
Cardinal	T5	5.00	0.80
L. Rosetta	T5	10.00	0.54
Granola	T5	15.00	0.20
Asterix	T5	10.00	0.46
BARI ALU 29	T5	7.00	0.34
Diamant	T6	9.00	0.18
Cardinal	T6	6.00	0.63
L. Rosetta	T6	9.00	0.29
Ganola	T6	15.00	0.31
Asterix	T6	4.00	0.31
BARI ALU 29	T6	7.00	0.28
	LSD	1.91	0.13
	Level of significance	**	**

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

During callusing of explants data was recorded when single shoot was regenerated from callus.

Very slow growth of shoot was observed during the experiment.

4.2.4. Days to shoot initiation

Effect of various potato genotypes on shoot initiation and length of shoot has been presented in Table 4. Maximum days (9.14 days) required for shoot initiation was recorded in L. Rosetta and a minimum day (5.33 days) s required for shoot initiation was recorded in Diamant.

The effect of different treatments on days required for shoot initiation has been presented in Table-5.The MS media containing T6 treatment (0.5 mg/l GA3 + 1.0 mg/l 2, 4-D+ 1.0 mg/l KIN) required maximum days (14.50 days) to initiate single shoot from callus induced from meristem and the medium containing T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 1.5 mg/l KIN) required minimum days (5.13 days) for shoot initiation.

The combined effect of different treatments and potato genotypes on days required for shoot initiation showed that L. Rosetta 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 Cardinal, L. Rosetta and Granola 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 Cardinal 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 Diamant and Granola was cultured in medium containing T6 (0.5 mg/l GA3 + 1.0 mg/l 2, 4-D+ 1.0mg/l KIN) and BARI ALU 29 was cultured in medium containing TS (0.5 mg/l GA3 + 1.0 mg/l 2,4-D+ 0.5 mg/l KIN). (Table 6).

4.2.5. 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 Granola at 30 DAI and the lowest shoot length (0.27 cm) was recorded in Diamant at 30 DAI (Table 4).Shoot length/ callus were significantly influenced by different concentrations of GA3, 2, 4-D and KIN at 14, 21 and 30 DAI (Table 5). The highest shoot length (2.42 cm) was found in T3 (0.5mg/l GA3 + 0.5mg/1 2,4-D+ 1.5 mg/I KIN) at 30 DAI whereas the lowest shoot length (0.53cm) was recorded in T2 (0.5mg/1 GA3 + 0.5mg/1 2, 4-0+ 0.5 mg/I KIN).

The combined effect of potato genotypes and different concentrations of hormones showed variations at different days on shoot length (Table 6). The maximum shoot length (5.88 cm) was recorded in Granola in T3 (0.5mg/l GA3 + 0.5mg/l 2, 4-D+ 1.5 mg/l KIN) at 30 DAI and minimum shoot length (0.20cm) was observed in L. Rosetta in T2 (0.5mg/l GA3 + O.5mg/l 2, 4-D+ O.5mg/l KIN) and Cardinal in T1 (0.5mg/l GA3 + 0.5mg/l 2, 4-D + 1.0 mg/l KIN) at 30 DAI. T3 was found most effective treatment among all treatments to initiate and regenerate single shoot from callus induced from meristem and Granola was the best genotype in response to hormonal treatment T3. No interaction effect on shoot length were observed by diamant in T2 (0.5mg/l GA3+0.5mg/l 2, 4-0+1.0mg/l KIN) and T6 (0.5 mg/l GA3 + 1 mg/l 2, 4-D + 1 mg/l KIN), Cardinal in T2 (0.5mg/l GA3+0.5mg/l 2, 4- D+1.0mg/l KIN) and BARI ALU 29 in T5 (0.5mg/l GA3+1.0mg/l 2, 4-D+ 1.0 mg/l KIN) .

4.2.6. Days to root initiation

Days required for rooting in response of the treatment applicant was found non-significant in this experiment. No root formation was recorded in Asterix when cultured in T2 (0.5 mg/l GA3). Diamant when cultured in T1 (Fresh media), T2 (0.5 mg/l GA3), T3 (1.5 mg/l GA3) and in Cardinal when cultured in T1 (Fresh media) as well as in Asterix when cultured in T1 (Fresh media).

Table 4: Effect of different potato verities on regeneration of single shoot/callus

Potato genotype	Days to shoot initiation	Shoot length (cm)			Days to root initiation
		14DAI	21 DAI	30 DAI	
Diamant	5.33	0.22	0.25	0.27	-
Cardinal	8.74	0.19	0.36	1.07	19.17
L.Rosetta	9.14	0.35	0.55	1.60	15.77
Granola	5.64	0.52	0.75	2.41	11.15
Asterix	7.58	0.58	0.59	1.38	-
BARI ALU 29	7.00	0.35	0.47	1.41	17.61
LSD	0.91	0.11	0.14	0.54	-
Level of significance	**	**	**	**	NS

Table 5. Effect of different hormonal treatments on regeneration of single shoot/ callus

Treatment	Days of Shoot initiation	Shoot length (cm)			Days to root initiation
		14 DAI	21 DAI	30 DAI	
T0		0.55	0.55	0.57	
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
LSD	1.02	0.12	0.15	0.59	
Level of significance	**	**	**	**	NS

Table 6: Effect of different hormonal treatments and potato varieties on regeneration of single shoot/callus

Potato Varieties	Treatment	Days to shoot initiation	Shoot length (cm)			Days to root initiation
			14DAI	21DAI	30DAI	
Diamant	T0	3.00	0.60	0.60	0.60	-
Cardinal	T0	2.00	0.50	0.50	0.60	-
L. Rosetta	T0	2.00	0.70	0.70	0.70	
Granola	T0	2.00	0.50	0.50	0.50	-
Asterix	T0	4.00	0.50	0.50	0.50	-
BARI ALU 29	T0	3.00	0.50	0.50	0.50	-
Diamant	T1	--	-	-	-	-
Cardinal	T1	7.60	0.14	0.20	0.20	-
L. Rosetta	T1	10.00	0.30	0.82	1.08	-
Granola	T1	6.00	0.36	0.50	0.62	-
Asterix	T1	4.00	0.56	0.98	1.48	-
BARI ALU 29	T1	6.00	0.40	0.50	1.26	-
Diamant	T2	-	-	-	-	-
Cardinal	T2	-	-	-	-	21.00
L. Rosetta	T2	19.00	-	0.20	0.20	20.00
Granola	T2	4.40	0.64	0.70	0.70	10.00
Asterix	T2	5.00	1.08	1.58	1.70	-
BARI ALU 29	T2	5.40	0.42	0.46	0.56	14.00
Diamant	T3	4.00	0.30	0.34	0.34	-
Cardinal	T3	13.00	0.16	0.26	1.68	14.00
L. Rosetta	T3	5.00	0.48	0.60	2.62	9.00
Granola	T3	3.00	0.50	0.60	5.88	5.00
Asterix	T3	2.20	0.54	0.82	1.94	0.00·
BARI ALU 29	T3	3.60	0.34	0.48	2.06	10.00
Diamant	T4	12.00	0.13	0.24	0.36	-
Cardinal	T4	14.60	0.00	0.36	2.60	22.00
L. Rosetta	T4	4.00	0.28	0.50	3.26	-
Granola	T4	9.00	0.38	0.50	3.52	20.00
Asterix	T4	10.00	0.44	0.52	1.98	-
BARI ALU 29	T4	12.00	0.32	0.38	2.48	22.00
Diamant	T5	13.00	0.30	0.30	0.34	-
Cardinal	T5	9.00	0.50	0.58	0.64	20.00
L. Rosetta	T5	10.00	0.36	0.56	0.96	-
Granola	T5	12.00	0.74	0.74	0.74	-
Asterix	T5	13.00	0.20	0.30	0.30	-
BARI ALU 29	T5	-	-	-	-	-

Table 6 (Cont'd)

Diamant	T6	-	-	-	-	-
Cardinal	T6	15.00	0.00	0.60	0.60	-
L. Rosetta	T6	14.00	0.30	0.50	0.70	20.00
Granola	T6	-	-	-	-	-
Asterix	T6	17.00	-	0.40	0.50	-
BARI ALU 29	T6	12.00	0.14	0.50	0.64	23.00
	LSD	2.50	0.29	0.37	1.15	
	Level of significance	**	**	**	**	NS

4.3. *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 in such a way 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 Diamant, Cardinal, L. Rosetta, Granola, Asterix and BARI ALU 29, respectively (Table 7). 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 found 0%, 60%, 40%, 80%,0% and 60% in Diamant, Cardinal, L. Rosetta, Granola, Asterix and BARI ALU 29 respectively as shown in Table 8.

Table 7: Survival rate of *in vitro* regenerated plantlets in growth chamber

Potato genotype	Number of plants transplanted in tray	Number of plants survived	Survival percentage (%)
Diamant	20	17	85
Cardinal	20	18	90
L. Rosetta	20	15	75
Granola	20	18	90
Asterix	20	15	75
BARI ALU 29	20	15	75

Table 8: Survival rate of transplanted plantlets in field Condition

Potato genotype	Number of plants transplanted	Number of plants survived	Survival percentage (%)
Diamant	15	0	0
Cardinal	15	9	60
L. Rosetta	15	6	40
Granola	15	12	80
Asterix	15	0	0
BARI ALU 29	15	9	60

4.4. Bio-assay through ELISA:

Successfully *in vitro* grown plantlet samples from each genotypes were send to TCRC, BARI Laboratory for conducting a serological identification of plantlets against major potato viruses PLRV, PVY, PVX, PVS and PVM through double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) protocol (BARI, 2012). The result of the ELISA test is given in Table 9.Similiar activities on ELISA test also done by Hussain in 2005.

Table 9: Detection of different potato viruses from selected potato varieties through DAS-ELISA

Genotypes	Antiserum used				
	PLRV	PVY	PVS	PVX	PVM
Diamant	-	-	-	-	-
Cardinal	-	-	-	-	-
L. Rosetta	-	-	-	-	-
Granola	-	-	-	-	-
Asterix	-	-	-	-	-
BARI ALU 29	-	-	-	-	-

CHAPTER 5

SUMMARY AND CONCLUSION

The experiment entitled "**Virus Free Plantlet Production of Potato (*Solanum Tuberosum* L.) through Meristem Culture"** was conducted at the tissue culture laboratory of BARI Agricultural Farm Debiganj, Panchagarh , during the period from March, 2015 to February, 2016. Three different sets of experiment were conducted and protocols were developed. The first experiment was conducted to investigate sprouting abilities of six potato varieties viz. Diamant, Cardinal, L. Rosetta, Granola, Asterix and BARI ALU 29, 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 varieties. 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 Bangladesh and was collected from Bangladesh Agricultural Development Corporation (BADC). Six potato varieties 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 in relatively short period of time. Among four treatments 400 ppm GA3 found most effective on sprout length, number of sprout per potato, number of sprout per eye in minimum days compared to other treatments in all six varieties. The best treatment (400 ppm) was applied to initiate subsequent sprouting to obtain large number 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) were used to study effects on six potato Varieties 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 was observed in all Varieties when cultured in

T0 (fresh MS media without any hormone combinations) whereas 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/I KIN) in different potato Varieties. The lowest survival percentage (40%) obtained by genotype BARI ALU 29 in T1 (0.5mg/l GA3+0.5mg/l 2, 4-D+0.5mg/I KIN). The texture of Cardinal and Asterix was non friable and other four genotype developed friable callus. Color of different Varieties developed four different colors. The colors of callus were whitish green, yellowish green, greenish and reddish.

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/12, 4-D + 1 mg/l KIN) produced callus and took maximum days (9.17 days) to initiate callus. Granola required maximum days (7.68 days) to initiate callus and Diamant initiated callus in very short period of time (3.66 days). Granola cultured in T5 (0.5 mg/l GA3 +1 mg/l 2, 4-D+ 0.5mg/l KIN) initiated callus in 15 days and Diamant 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 Cardinal whereas minimum callus size was recorded (0.29 cm) in Diamant. 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 (0.31cm) 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 Cardinal 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 Diamant 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/1 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 Granola 30 DAI and shortest shoot length (0.27 cm) was recorded in Diamant 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 Varieties Granola was the best genotype and produced maximum shoot length (5.88 cm). 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 Cardinal and Granola whereas lowest in L. Rosetta, Asterix and BARI ALU 29. When plantlets grown in trays were transferred to field condition where survival rate greatly varied from 0 to 80%. Potato Varieties which did not survived in field

condition are Diamant and Asterix whereas maximum survival rate was recorded in Granola.

The result of present investigation indicated that 400 ppm GA3 can be sprayed to get sprouts of desirable amount and Granola 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 Varieties Granola 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 all Varieties gave virus negative results.

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

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APPENDICES

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

Components	Concentrations (mg/L)
Macronutrients/ Major salts	
$KN0_3$	1900
NH_4N0_3	1650
$MgS0_4 \cdot 7H_2O$	370
$CaCI_2 \cdot 2H_2O$	440
KH_2P0_4	170
Micronutrients/ Minor salts	
$H3^B03$	6.2
$MnS0_4.4H_2O$	22.3
$ZnS0_4.4H_2O$	8.6
KI	0.83
$Na_2Mo0_4.2H_2O$	0.25
$CoCI_2.6H_2O$	0.025
$CuS0_4.5H_2O$	0.025
Iron Sources	
$FeS0_4 \cdot 7H_2O$	27.80
Na_2EDTA	37.30
Vitamin and Organic Nutrients	
Thiamine (HCI)	0.1
Niacine	0.5
Glycine	2.0
Pyrodoxine (HCI)	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	Degree of freedom					
		Days to shoot initiation	14 DAI	21 DAI	30 DAI	Days to root initiation
Factor A	5	2.88**	173.102**	1.067**	431.947**	64.773NS
Factor B	5	1.013**	50.262**	5.547**	89.075**	60.76NS
AB	25	0.349**	21.364**	1.373**	64.405**	16.829NS
Error	144	0.053	5.986	0.267	9.128	2.436

** = Significant at 1 % level
* = Significant at 5% level
NS= Non Significant