CALLUS INDUCTION AND VIRUS FREE POTATO MINI-TUBER PRODUCTION THROUGH MERISTEM CULTURE

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CERTIFICATE

This is to certify that the thesis entitled **'CALLUS INDUCTION AND VIRUS FREE POTATO MINI-TUBER PRODUCTION THROUGH MERISTEM CULTURE'** submitted to the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology, embodies the results of a piece of bonafide research work carried out by Sayma Serine, Registration No. 16-07569 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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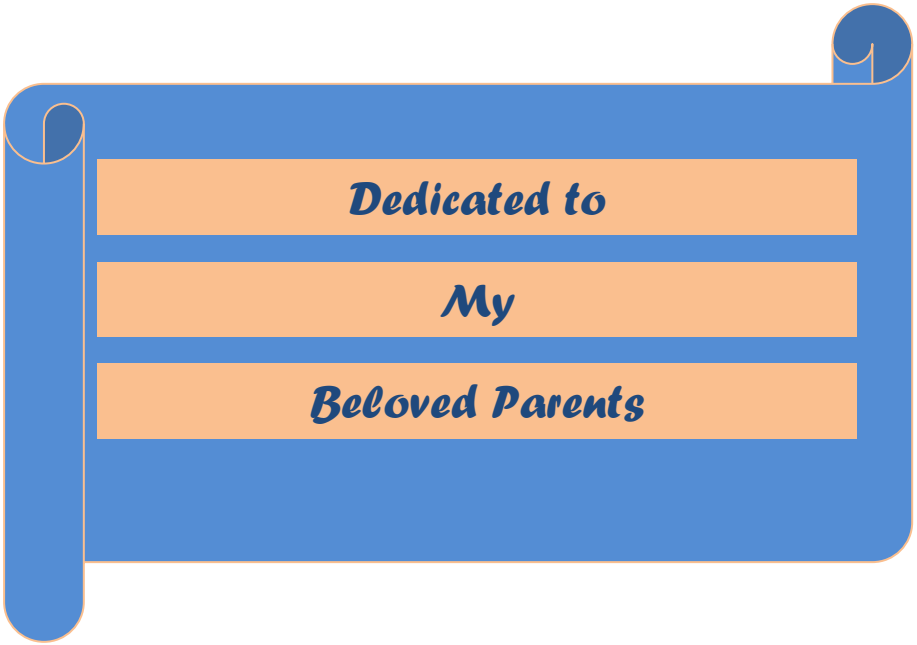
Dedicated to
My
Beloved Parents

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ABSTRACT

The experiment was carried out by taking the facilities of the laboratory of Biotechnology Department, SAU and the laboratories of Plant Tissue Culture, Molecular Biology and Plant Virology, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207, during the period of July, 2016 to August 2017. The experiment was conducted with a view to establish a protocol for *in vitro* production of virus free mini-tubers, using potato meristem as explant collected from potato sprouts of three popular potato varieties viz. , Granola, Diamant and Cardinal. Plant growth regulator GA3 (400 ppm/L) was used to assess the influence on sprouting ability. Among the selected potato cultivars, sprouting efficiency was observed at 400ppm/L GA3 treatment within short period of time in Granola variety. The effect of different combination and concentration of 2, 4-D, BAP and IBA was used along with fresh MS media to inoculate meristems of potato sprouts. Six different combinations of 2, 4-D, BAP and IBA with a control treatment were used in the experiment. The several concentrations of 2, 4-D (2, 4-D: 0.125, 0.25, 0.37, 0.50, 1.0, 2.0 mg/L) BAP (BAP: 0.5, 1.0, 1.5, 2 mg/L) and IBA (IBA: 0.25 and 1.50 mg/L respectively) in the experiment. The maximum (0.74 cm) callus size observed in Granola within a very short (2 days) period of time while treated with T_3 (0.25 IBA+0.25 2, 4-D +1.0 BAP). Granola gave the highest (9.32 cm) values in respect of length of shoot, number of shoots/plantlets (4.33) and root length (7.14 cm) along with maximum (15.00) number of leaves/plantlets whereas Cardinal gave minimum (3.10 cm) values in respect of length of shoots/plantlets, maximum (1.33) number of shoots/plantlets and number (4.00) of leaves/plantlets was found in Diamant variety. Granola showed maximum (8.44 cm) length of root while minimum (1.87 cm) length of root was found in Cardinal variety among the selected cultivars. Over all Granola showed better performances from meristem tissue culture and plants were found normal and free from potato viruses as they were tested through ELISA test.

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CHAPTER 1

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the world's significant tuber food crops, it is full-grown in large areas all around the world and occupied the fourth importance next to rice, wheat and maize. Potato is the member of the family solanaceae and pertain to the genus *solanum* and is the most important foodstuff of the world, with annual production nearer 300 million tons (CIP, 2007). Potato is propagated vegetatively through tubers; it is originated in South America. Potato is used for human consumption and animal feed. It is used as a fastener food in many countries of the world, but generally as a vegetable in Bangladesh (Hussain, 1995). In the world, potato is cultivated 53,666 thousand ha of land and production is 752,632 thousand tons and in Bangladesh, potato cultivated in 4, 30,255 ha of lands and production is 82, 05,470 metric tons. Potato is cultivated in 23 major growing areas in Bangladesh.

The potato tuber is an excellent source of carbohydrates, protein and vitamins (Mac Gillivary, 1953). Nutritionally, the tuber is rich in carbohydrates or starch and is an tremendous supplier of protein, vitamin C, vitamin B, potassium, phosphorus, and iron. Most of the minerals and protein are concerted in a slim layer underneath the skin, and the skin is a source of food fiber resource of starch and alcohol. Potato is highly rich in good amount of essential amino acids likely leucine, tryptopan and isoleucine (Khurana and Naik, 2003). Potato also contains a diversity of phytonutrients that facilitate antioxidant activity. The Irish potato is one of the most extensively grown tuber crops in the globe that contributes enormously to human nutrition and food refuge (Steven, 1999, Karim *et al.,* 2010).

In contrast to other agricultural crops, the seed cost of potato crop growing is much privileged. Bangladesh Agricultural Development Corporation (BADC) reported that the seed cost of potato is accountable to 30 - 40% of total fabrication cost. Bangladesh imported high yielding foreign potato varieties at

the cost of over US $ 150.00 per quintal which occupy bulky amount of foreign currency every year.

According to (DAE) farmers grew HYP tubers approximately in 4,92 lack ha of land during the (2017-2018) production year, which was 1.4% less than the 4.99 lack ha of the previous year. It is anticipated that the yield of potato tuber production was 80-85 lack metric tons. in Bangladesh which was occupied the 7th position in care of potato production worldwide (BBS, 2017-2018). Utmost cultivation and production of potato were seen in the district of Dinajpur, Thakurgaon, Bogra, Joypurhat, Nilphamari and Munshigonj in Bangladesh.

In the last few decenniads, a number of dozens of high yielding varieties (HYV) of potato were imported to Bangladesh and tried experimentally underneath restricted conditions previous to being recommended for general cultivation (Islam *et al*., 2003).

High yielding foreign potato varieties considerably improved the yield of potato crop in our country but all together it results new viral troubles like *PLRV, PVY* and *PVX* which have been estimated 10-90% yield losses in Bangladesh. The virus-free clone created more vigorous haulm and about 10% higher yields, imposed to more tubers more willingly than large ones (Karim *et al.,* 2011). Zhang (1995) reported 40% yield increase in potato using virus free tuber seeds.

In many on the rise countries together with Bangladesh, yields of root and tuber crops are significantly attenuated below their potential due to numerous seed-borne diseases and vermins. In vegetative propagated potato crops, once methodically infected with a viral disease, the pathogen is passed from one vegetative generation to next generation. There are more or less 23 virus and virus like organisms that are source of diseases in potato. Khan (1981) concluded that a solo plant of potato variety has been infected with about four to five viruses. The occurrence of viral disease is a vital reason for low yield of potato varieties in our country (Siddique and Hussain, 2012).

Yield losses are straightforwardly proportional to the severity of virus infection. Per hectare yield could be doubled by the use of vigorous and sound seed (F.C. Bawden and B. Kassanis, 1965). Biotechnological techniques could be taken in consideration to solve this problem and comprehend great advantage to potato farmers.

Plant renewal from cell and tissue culture symbolize an crucial component of biotechnology which is used to improve not only the existing cultivars, but also for the creation of novel plants in a moderately short time than conventional breeding (G.A.E., Khadiga, S.M. Rasheid and M.M. Khalafalla, 2009). Numerous tissue culture protocols have been taken for massive amount of potato cultivars (Hashem *et al.*, 1990). Tissue culture may causes distinction either in challenging or useful for horticulturists and plant breeders and occurrence of adventitious plant regeneration or long-standing callus production is so high (Kaeppler, S. M., H. F. Kaepler and Y. Rhee, 2000).

Production of virus free potato through meristem culture protocol has been established in many crops (Bhojwani and Razdan, 1983). Newly *in vitro* preservation protocol for virus-free potato is also long-established (Tiwari *et al.*, 2013). A number of researchers deliberate the improvement which has been made in callus induction and plant renaissance of potato (Khadiga, *et al*., 2009; Shirin, *et al*., 2007; Khalafalla, *et al*., 2010).

Tissue culture process are highly competent in bringing genetic changeability and producing plants with narrative characters. This indicates, tissue culture submission could be the practicable alternatives in rising new cultivars apart from producing virus free planting stocks and flourish heterozygous segregates.

Objectives:

Taking into consideration facts and points that mentioned as background of this study was deliberate to accomplish the following specific objectives

- To develop reproducible protocol for producing of virus free potato seed tubers.
- To produce and multiply virus free mini-tuber through meristem culture.
- To assay true potato plants growing from mini-tuber against major potato viruses.

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CHAPTER 2

REVIEW OF LITERATURE

The potato *(Solanum tuberosum* L.) as a vegetative 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

Conventional methods of crop enhancement and safeguarding of breeding materials are prolonged and less effective. The plant tissue culture technology has been developed as a modern and most prevailing tool for crop upgrading (Razdan and Cocking, 1981) and received wide consideration of the modern scientists (Larkin and Scowaroft, 1982). Plant tissue culture can play an significant role over the conventional methods of crop improvement especially in the genetic manipulation of crop plant species (Reddy and Kishore, 1982; Zapata *et al.*, 1987).

Carlson (1975) the nonconventional methods namely cells and plant tissue culture have opened up numerous new potential for manipulation to induce genetic variations and selection of advantageous traits. However, plant regeneration from in vitro cultures is a precondition of many plant genetic alteration techniques.

Dodds and Roberts (1982) regeneration from different explants (leaf, stem, internode, roots, etc.) on defined nutrient media under aseptic conditions is the base of plant tissue culture. Callus is the undifferentiated loosely prearranged parenchymatous tissues nourished when an explant of a plant species is grown on defined culture medium. And subsequent expansion of root, shoot and ultimately a whole plant from these undifferentiated cell mass is regeneration

Binsfeld (1999) observed that the systematization of genetic breeding programs happening in the beginning of the of the century with the innovation of the basic principles of mendalian segregation which set out the basic laws of genetic heredity. Since then, the appliance of genetic principles to the progress of the plants with superior agricultural performance, through the application of most varied methods, has been systematic. At the same time, there has been extensive progress in *in vitro* plant cell and tissue culture techniques.

Tissue culture practice is now used expansively in many national and international organizations such as, Bangladesh Rice Research Institute (BRRI), Bangladesh Agricultural Research Institute (BARI), Bangladesh Sugarcane Research Institute (BSRI), Bangladesh Jute Research Institute (BJRI), Dhaka University (DU), Bangladesh Agricultural University (BAU), Rajshahi University (RU), Chittagong University (CU), International Potato Centre (CIP), Bangladesh Institute of Nuclear Agriculture (BINA), International Rice Research Institute (IRRI), where programs of crop upgrading are in improvement for the development of different crops.

2.2. Plant tissue culture and plant breeding

Binsfeld (1999) plant breeding aims at rising new cultivars that are adapted to the constant and high yield cultivation conditions requisite by high quality production. From the methodological point of view, plant breeding is applied genetics and has been considered the art and science of varying plants genetically for human utilization. Even though the extraordinary contribution of

the conventional methods to plant breeding, there has been a harmony that significant gains cannot be expected from choice by these processes only.

Binsfeld (1999) explained that traditional methods developed innovative cultivars until the mid 1980s. Since then, plant breeding has developed a number of techniques based on plant tissue culture and molecular biology. These techniques became imperative tools to help breeders look for the allelic diversity needed in a breeding program, enabling them to go above the primary genetic tool used in classic plant breeding and to integrate the new alleles into the genome to find out the required characteristics of the culture.

2.3. Concept of meristem culture

The apical meristem mutually with one to three young leaf primordia, measuring 0.1-0.5mm, has been referred as meristem-tip. The allotment of viruses in plants is irregular. In infected plants the apical meristems are in general either free or bring a very low deliberation of viruses. Meristem-tip culture although mainly used for virus eradication, it has also enabled plants to 77%, depending on the cultivar and possibly also on the virus filter in the parent stock. Mericlones once tested negative to the potato viruses with ISEM sustained to remain negative upon succeeding sub culturing over a period of 2 years.

2.4. Effect of meristem size for meristem culture

Shakya *et al.* (1992) in the course of 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 diverse 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 different combinations. Continued existence of the meristem was subjective by

the culture medium, kind and size of the meristem excised. Superior survival percentage (40.5%) was gained from apical meristems in comparison to the lateral meristems (25.9%). It was also mentioned that the lesser sized meristems (0.11-0.25 mm diameter) were more efficient than the larger ones in producing virus-free potato plantlets.

In an experiment carried out 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 dissimilar supplements of growth hormones (kinetin and GA3). Basal medium did not maintain survival and growth of small sized (0.2 mm) meristems. Addition of 0.5 mg kinetin/litre alone to the medium was not effective to raise survival and percent regeneration. MS medium supplemented with 0.2 mg kinetin + 10 mg GA311itre was most appropriate 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

A paper was available by Jing in (2004) stating an experiment carried out on Seed tubers of potato cv. Favorita which were subjected to two GA3 treatments to examine their effects on seed germination rate. Seed tubers treated with GA3 solution (20 mg/l) reserved in dark room followed by spraying of GA3 (20mg/l) after 7 days resulted 96.75% germination rate. The seed tubers sprayed with GA3 solution (20 mg/l) for 1 hour and set aside them in a dark room showed 98.75%. The culture circumstances of all treated seed tubers in the dark room were the same.

2.6. Callus induction and plantlet regeneration

Shahab-ud-din *et al.* (2011) have carried out an experiment to explore the effects of different concentrations of plant growth regulators and their combinations on callus induction of potato (*Solanum tuberosum* L.). The

explants of potato tuber were cultured on ready made MS medium that was supplied with different concentrations of 2,4-D, NAA and BA for callus initiation. The concentration of sucrose was 3% W/V stage and the pH of the medium was accustomed to 5.7 before the adding up of agar 8% W/V. At first the explants were dissected out aseptically and after dissection the explants were inoculated to the media (with various levels of hormones), then incubated at 27±2ºC in the culture room. Amongst the treatments 2,4-D at different concentrations formed different degree of calli but moderately a massive amount of calli were produced on MS medium supplemented with 2,4-D alone at 3.0 mg/L. Also NAA and BA with diverse concentrations formed significant degrees of callus but the degree of callus was most excellent at higher concentrations of NAA and BA. 2,4-D in combination with BA at 2.0 mg/L equally formed extensive amount of callus. In case of NAA and BA mixture the degree of callus development was best at concentration 1.0 mg/L each. So according to the above termination it was concluded that 2,4-D is the best option for induction of callus among the other hormones used in the study.

Khalafalla *et al*. (2010) in an experiment indicated that the procedure of plant rejuvenation from callus culture of potato (*Solanum tuberosum* L.). Calli were commenced from 1.0 cm^2 tuber fragment of potato cultivar Almera on MS medium supplemented with different levels (1.0-5.0 mg/L) of 2,4-D. The 100% explants formed nodular calli within 7- 12 days on MS medium when supplemented with 2.0-5.0 mg/Lof 2, 4-D. Induced calli were differentiated into shoot-primordia when these were subcultured on MS medium supplemented with 1.5-5.0 mg/L of thidiazuron (TDZ) and 2.0-5.0 mg/L6 of benzyladenine (BA). The superior result for number of shoot/callus (3.3 ± 0.3) and the longest shoot (0.8 ± 0.1) were obtained in case of using TDZ at 5.0 mg/L. Callus resulting shoots were rooted most efficiently in undiluted MS medium that contains 1.0 mg/LIBA. The achievement of plant tissue culture for in vitro culture of potato was optimistic by adaptation of the plantlets in the greenhouse conditions. Regenerated plants were morphologically identical with typical leaf

character and growth pattern.

Hoque *et al.* (2010) examined *in vitro* microtuber development potentiality of potato was inspected to set up a rapid disease free seed production system in potato. MS medium supplemented with 4 mgL-1 of kinetin displayed the best performance in respect of numerous shoot renaissance and microtuber construction. Simple MS medium was noteworthy to produce any micro tuber under in vitro condition. Dark condition responded better in case of tuberization than light condition. Among the three diverse explants (nodal segment, sprout and shoot apex) nodal cutting showed the best performance on days to microtuber development and typical weight of microtuber. Superior tuberization in in vitro condition were obtained with the combination of treatment MS + 6% sucrose + 4 mgL-1 kinetin among the parameters under study.

Hussain, I. (2005) carried out a study *in vitro* response and its relationship with dissimilar varieties, explants and medium were investigated in potato (*Solanum tuberosum*). Direct In vitro regeneration practice from varied explant source is a precondition for alteration studies. Three potato cultivars viz., Cardinal, Altamash and Diamant were chosen for in vitro responses. High regeneration and morphogenic possible of unlike explants i.e., shoot tips, nodes, leaf discs and internodes have been examined for direct regeneration. Basal media Murashige & Skoog was used with different growth regulators in combinations of benzyl adenine and indoleacetic acid were supplemented. Statistical analysis reviewed that explants resource had considerable effect on through regeneration and the nodal explants had greatest regeneration. The number of shoots obtained from node was 17.6 from Cardinal followed by Diamant 14.3 and Altamash 9.0. Shoot 7 apices also evolved in shoot regeneration relatively better than leaf discs and internodal explants but lesser than from nodes. Most appropriate medium was MS with 2.0 mg/L BAP and IAA @ 0.5 mg/L generous utmost regeneration. It was also pragmatic that interface of cultivars with explant and media is vastly significant at P 1.0%.

2.7. Effect of Hormonal combinations and culture environment

A research was conducted by Nagib *et al.* (2003) to expose out the best hormonal combination for meristem culture and *in vitro* regeneration in three cultivars i.e. Diamant, Cardinal, Multa and Lalpakri. Among diverse types of hormonal combinations 0.5 mg/l GA3 and 0.04 mg/l KIN combination was originate to be best medium for the major establishment of meristem.

The crucial developed meristems were sub cultured on MS medium and MS media containing BA and IBA individually or combinations. Allowing for all treatments singly use of IBA (0.5 mg/l) was recommended for suitable shoot and root enlargement from primary meristem. In the conduct experiment it was also viewed that 2.0 mg/l GA3 was the best media for shoot induction and combinations of GA3 (0.1 mg/l) + KIN (0.1 mg/l) was most effectual for high incidence of root formation for deliberate varieties. Considerable yield enhancement was also observed in meristem resultant plants over their source plants Nagib *et al.* (2003).

An experiment was conducted by Sheng *et al.* (2003). In this experiment stem tip meristems in the company of 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) + 1M (0.1 mg/litre) + GA3 (0.1 mg/litre). They were after that 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 extent of 12, 14 and 16 h. The most favorable temperature and day length were 28° C and 14 hrs/day for plantlet renewal. Supplementation of IBA or NAA to the MS medium at the rate of 0.1-0.5 mg/litre was noticed advantageous to plantlet regeneration and the effect of IBA was found improved. Supply of IBA at 0.5 mg/litre amplified the regeneration rate by 9.9-15.4% as compared with the manage and reduced the mean time for plantlet development by 3.3-8.5 days. An elevated concentration (1.0 mg/litre) of the auxins resulted in the formation of abundant calluses on the explants.

Badoni and Chauhan (2009) conducted an trial on Potato (*Solanum tuberosum*) cultivar Kufri Himalini where meristem tips were cultured on MS medium

supplemented with unlike 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) that were effected *in vitro* proliferation of potato. It was originate that lesser concentrations of auxin (0.01 mg/l NAA) aid with Gibberelic Acid (0.25 mg/l GA3) are finest for maturity of complete plantlets and reproduction from meristem tips.

Zaman *et al.* (2001) noticed the effects of three dissimilar 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 manufacture of virus free potato plantlets. Maximum plantlet height (8.3 cm), largest number of nodes/plantlet (7.3) and uppermost number of leaves/plantlet were reported at 0.5 mg/l NAA followed by 1 mg/l IBA. Whereas not narrow number of roots/plantlet (23.7) as well as the earliest micro tuber formation (17 days after transplantation) were recorded at 1.0 mg/l IBA followed by 0.1 mg/l NAA.

In another experiment Peyman *et al.* (2004) meristem explants of potato genotypes were *in vitro* cultured on fluid and firm 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 shifted 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 momentous 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 showed the most excellent combination for shoot initiation. It was also inspected that, NAA at 0.7 mg/litre initiated the highest shoot regeneration which was 76%.

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 manufacture of virus-free plantlets were deliberate by Ghaffoor *et al. in* (2003). The parameters taken to observe were plantlet height, number of node/plantlet, number of leaves/ plantlet, root length and number of roots/plantlet. The recorded plantlet

height (9 ern) 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 amount of leaves/plantlet was found maximum (6.143) with IAA at 0.25 mg/litre. The incidence of PVX (potato virus X), PVY (potato virusY) 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 transited by PVY and PLRV.

Orthogonal design with 3 factors at 5 levels was adopted to display most select plant hormone combinations that could provoke stem segments to distinguish 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 fragments of virus-free seedlings *in vitro* of potato cv. Super White were used as the explants and the MS medium was used as crucial culture medium. Effects of the 3 plant hormones on callus discrimination was ranked as NAA>SA>GA3. The optimal combination was reported 0.25 mg NAA + 1.5 mg SA + 7 mg GA3/litre Yushi *et al.* (2004).

Xian (2005) studied an experiment where stem portions with a distinctive node from *in vitro* plants of Youjin were used as explants. These explants were inoculated on MS (Murashige & Skoog) medium supplemented with diverse combinations of NAA, BAP (6-benzylaminopurine) and GA3 (gibberellic acid) and cultured for 20 days beneath a photoperiod of 12-16 h, and callus initiation in potato. In addition, the effects of cultivar and explant were appreciated on callus organogenesis. Callus stimulation on internode explants was tested using MS medium supplemented with blend of 1, 2 or 3 mg 2, 4-0/litre and 0.00, 0.01 or 0.10 mg kinetin/litre. Analysis of variance showing a significant effect of 2, 4-0 and kinetin appliance, and their interface, on the frequency of callus initiation and amount of roots on the callus. The effects of kinetin concentration and kinetin x 2, 4-0 interface were significant on the establishment time of callus orientation and volume of callus. Though, the effect of 2, 4-0

concentration alone on these variables was originate insignificant. The effects of cultivar and explant on callus stimulus in leaf and internode explants of potato cultivars (Agria, Cosmos, Sante, Concord, Ajax and Oiamant) were deliberate. Leaf and internode explants were excavated on MS medium, supplemented with 5 mg 2, 4-0 and 0.25 mg kinetin/litre. Analysis of variance stated significant effect of cultivar and cultivar x explant interaction on callus volume, while the effect of explant was insignificant. The effect of cultivar, explant and their interface on frequency of callus orientation was not significant, while the effects of these factors on the instigation time of callus instruction was significant. In conclusion, the effect of light on callus induction in leaf and internode explants was sought. Callus was typically induced in leaf explants under not in bright conditions, but was induced in internode explants under both dark and brightness conditions. Also in the renaissance stage, the effects of cultivar and explant on callus organogenesis were calculated significant.

2.8. Shoot development from meristem culture

The shoot apical meristem is accountable for primary shoot expansion, whereas sideway branching is initiated by the enlargement of axillary meristems formed in the axils of leaves, axillary meristems come up post embryonically and are derived either not indirectly from the meristematic cells of the shoot apical meristem of potato *(Solanum tuberosum)* Sussex (1955).

2.9. *In vitro* plant regeneration and multiplication

The abundant and not similar tissue culture methods were done in potato. These methods permit speedy multiplication of potato clones Ahloowalia (1994).

It was observed that producing mini-tubers from *in vitro* plantlets deliver sooner multiplication rate in seed tuber production programs and shrinkage the number of requisite field generations Imma and Mingocastel (2006).

Balali *et al.* (2008) deliberate the mini-tuber production in the plantlets that sprouted from virus free sprouts and a genotype of the identical cultivar (Marfona) originated from apical meristem. The consequences showed that the number of mini-tubers per plant was advanced 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 gained through meristem culture. In the study to grow plantlets which were obtained in meristem culture practice, they cultured sole node in the MS solid media containing NAA and BAP. They reviewed that the best media for single node culture is MS media exclusive of any growth regulators.

Petioles, internodes and leaf explants in combination with different plant growth regulating hormones, particularly special concentrations of zeatin riboside (ZR), were experienced in an trial conducted by Zel and Medved (1999). It was observed that the shoot regeneration was most victorious on callus originated from internode tissue cultured on instruction medium supplemented with 2.5 mg ZR, 0.2 mg NAA, 0.02 mg gibberellic acid (GA3)/litre for 2 weeks and then shifted to a shoot induction medium that contains 2.5 mg ZR llitre. In a contrast of the regenerative potential of Igor with that of Desiree, Igor had of poorer quality and slower regeneration and formed smaller amount and shorter shoots. However, the protocol established was concluded suitable for shoot regeneration for use in Agrobacterium-mediated transformation of Igor.

A continued regeneration system was narrated by Rodriguez *et al.* (2000) using leaf explants of potato cultivars Diacol Capira (DC) and Parda Pastusa (PP). The effect was sought of different ratios of auxins and cytokinins combined 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 conducted 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, initiating green and morphologically typical plants.

Three potato cultivars, Cardinal, Altamash and Diamont were preferred for *in vitro* repercussion in an experiment. High regeneration and morphogenic prospective of different explants i.e., shoot tips, leaf discs, nodes and internodes have been examined for through regeneration. Basal media was Murashige and Skoog and several hormonal combinations of benzyl adenine and IAA were supplemented.

Statistical analysis reviewed that explant resource had momentous effect on direct regeneration and the nodal explants had not minimum regeneration. The number of shoots achieved from node was 17.6 from Cardinal followed by Diamont 14.3 and Altamash 9.0. Shoot apices also resulted in shoot regeneration moderately superior than leaf discs and internodal explants but lesser than from nodes. The most appropriate medium was reported MS with 2.0 mg/litre BAP and IAA at 0.5 mg/litre that gives maximum regeneration. It was also found that interaction of cultivars with explant and media was vastly significant at 1.0% Hussain *et al*.(2005).

Holmes (1948), Limasset (1949) and Kassanis (1957) have illustrated that 'Imperfect allocation of virus in plant body and the nearer to the tip of plant the minor the content of virus'. Since then this fact has developed into manufacture method of virus-free clone by grafting and cutting of the plant's tip by the Holmes' research (1948, 1955), and with this decision being associated by tissue culture method under the research finding of Morel (1955) of France, eventually resulted in the virus-free meristem culture which is a long-awaited get through in the treatment of virus disease-the disease which revolted the challenge of many researchers, for so long.

2.10. Field performances of different potato varieties

Chehaibi *et al.* (2013) conducted an experiment in the research station of the higher institute of agronomy of Chott-Mariem in the Sahel region of Tunisia with two varieties of potato: Alaska and Safrane, were instinctively planted at two diverse depths. The consequences showed that for tuber yield of Alaska variety was more fruitful than the Safrane variety with growing yield of tubers.

Jatav *et al.* (2013) trialed an experiment was at Central Potato Research Station, Jalandhar to estimate potato cultivars viz. Kufri Jyoti, Kufri Jawahar, Kufri Bahar, Kufri Sutlej, Kufri Pukhraj, Kufri Pushkar, Kufri Surya and Kufri Gaurav at four N levels and outcome exposed that Kufri Gaurav reported not minimum yield, agronomic effectiveness and net resume at all the levels of nitrogen followed by Kufri Pushkar and Kufri Pukhraj. Kufri Surya yielded bare minimum with slightest agronomic efficiency at all the levels of nitrogen.

Jovovic *et al.* (2012) conducted an experiment to find out the distinctions among genotypes for potato yield and filed the peak yield was calculated at variety Agria (30.0 t ha-1), whilst the lowest at Riviera (24.6 t ha-1) and suggested that Agria variety was favorable for yield of potato tuber.

Abbas *et al.* (2012) directed an experiment on two potato genotypes for dealing out and yield quality traits. Significant differences in all the worth parameters and a choice of characteristics were observed, while the genotypes; 394021120, 9625, Kiran, NARC 2002-1, NARC 1-2006/1 and VR 90-217 outcomes the highest results on the subject of yield and quality of potato tubers except Kiran, which has a high yield but stumpy quality characters. The tuber sizes and weight was also significantly different among genotypes excluding weight of big size tubers.

Karim *et al*. (2011) studied with ten exotic potato varieties (var. All Blue, All Red, Cardinal, Diamant, Daisy, Granola, Green Mountain, Japanese Red, Pontiac and Summerset) to established in the experiment field for presenting yield performance of tuber numeral per plant and tuber weight per plant from

10 indiscriminately selected potato plants of every variety. The uppermost tuber number (57.52) per plant was filed in var. Daisy and the lowest number of tuber (8.82) per plant was recorded in red varieties. Conversely, total tuber weight per plant was maximum (344.60 g) recorded in var. Diamant and total tuber weight per plant was minimum (65.05 g) recorded in var Pontiac. All blue varieties displayed the most potential yield in this experiment.

Sharma and Sarjeet (2010) was experimented the production potential of small (10 g) seed tubers of 4 potato varieties of hills viz., Kufri Shailja, Kufri Kanchan, Kufri Giriraj and Kufri Jyoti at three plant densities viz., 83,333; 111,111 and 166,666 plants/ha. Plant vigor in esteem of height, number of shoots and compound leaves was exaggerated appreciably by the changeable plant densities as well as by the genotypes. Days to foliage development were unbiased by the plant densities but varieties differed significantly. Number of total and seed size tubers were utmost in Kufri Shailja (6.09 and 3.33 lac/ha, respectively) at the maximum plant density (166,666 plants/ha) and least in Kufri Jyoti (2.36 and 1.00 lac/ha respectively) at the minimum plant density. Likewise, total and seed size tuber yields were greatest in Kufri Shailja (369.0 and 175.8 q/ha) at the maximum plant density. Potato yields were lowest amount in Kufri Jyoti (242.6 q/ha) at minimum plant density, while seed size tuber yield was minimum (55.3 q/ha) in Kufri Giriraj but at per with Kufri Jyoti. A field experiment was carried out by Pandey *et al.* (2009) to evaluate the achievement of 3 indigenous potato (*Solanum tuberosum*) processing cultivars (Kufri Chipsona 1, Kufri Chipsona 2 and Kufri Jyoti) and 2 foreign cultivars (Atlantic and FL 1533) in Agra, Indore, Jalandhar, Kufri, Modipuram and Ooty, Uttar Pradesh, India. The utmost total tuber yield, processing-grade tuber yield, tuber dry matter content was filed for two exotic cultivars.

Luthra *et al*. (2006) reported that Kufri Arun is a standard maturing, main season, high yielding table potato variety proper for cultivation in north Indian plains. It is a clonal collection from the cross between Kufri Lalima and MS/82-797. Its plants are high and vigorous with field confrontation to late blight. Its tubers are red, oval with small-minded to medium eyes and creamy-light yellow

flesh, and having good keeping worth. It is fertilizer responsive and proficient of yielding 350-400 q/ha under most favorable agronomical practices.

Alam *et al.* (2003) characterized fourteen foreign varieties of potato (*Solanum tuberosum*) namely Mondial, Granola, Cardinal, Ailsa, Petronese, Morene, Diamant, Cleopetra, Binella, Dheera, Multa, Kufri Sindhuri, Heera, Chamak and a local check (Lal Pakri) under Bangladesh condition. The yields ranged of exotic varieties were 19.44 to 46.67 t ha-1. Variety Ailsa grew the maximum yield (46.67 t ha-1) which was followed by Cardinal and Mondial.

Hossain *et al.* (2003) observed that the yield contributing characters of varieties were not similar significantly. Highest yield (27.31 t ha-1) was gained from the variety Akira and it was alike to Jaerla (26.30 t ha-1) and these two varieties out yielded the check variety Diamant (22.81 t ha-1). The varieties Baraka, Jaerla, Bintje, Midas, Ultra, Akira, Dura, Granola, Futuri and Diamant yielded more than 20.00 t ha-1. Most of the varieties possibly incapable to prove their full yield potential due to the new environment of their first generation in Bangladesh.

CHAPTER 3
MATERIALS AND METHODS

All the materials and methods were used to facilitate the study have been presented in this chapter. It comprises a short narrative of the research, materials used, design of the experiment, data collection system and data analysis procedures.

3.1. Experimental site

The research experiment was carried out in Biotechnology Department and Plant Tissue Culture Laboratory and at Molecular Biology and Plant Virology laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka,1207.

3.2. Experimental duration

The experiment was conducted from July, 2016 to August 2017 to attain *in vitro* rejuvenation of meristem cultured plant materials of three potato varieties.

3.3. Variety Selection and Collection of Seed tubers

Three BARI released popular potato cultivars (Table-1) were selected to conduct this study. The cultivars name used their origin are given below in table 1.

Table 1: Name and origin of 3 potato cultivars used in the present study:

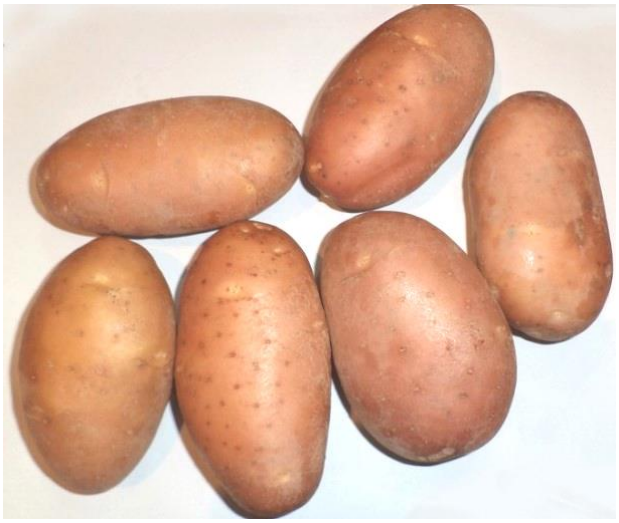
Sl. No.	Cultivar Name	Accesion No. (BD)	Origin
1	Granola	BARI Alu-13	PGRC,BARI
2	Diamant	BARI Alu-7	HRC, BARI
3	Cardinal	BARI Alu-15	HRC, BARI

3.4. General experimental protocol

Primary plant materials of three potato cultivars (Figure-1) were treated with GA3 to obtain sprouts where meristem tips composed. Meristem tip of about 0.2-0.5 mm length was used as primary explant for *in vitro* renaissance of callus under dissimilar hormonal treatments. Solitary shoot initiates from callus was

sub cultured in MS medium without or with diverse levels of GA3 concentrations. Additional subculture in MS medium with hormones appropriate for rapid multiplication of plantlets was done.

A **B**



C

Figure 1. Primary plant materials of selected cultivars Granola(A), Diamant(B) and Cardinal(C)

3.5. GA3 Treatment and collection of explants

Each and every potato cultivar was treated with GA3 to grow sprout. Potatoes were chosen cautiously then used to carry out this experiment. Comparable sized potato of each cultivar was taken. Prior to GA3 management potato varieties were soothingly washed with liquid detergent (Twin-20) and washed carefully in running tap water without losing potato skin to eliminate all kinds of dust. After that all of them were refined with distilled water followed by located on blotter paper and then dried up in usual room temperature to take away extra moisture on potato skin. Different potato varieties of identical size and weight were elected for spraying Two (2) concentrations of GA3 treatments (0.0 and 400 ppm). Application of GA_3 at different concentration found more or less similar result experiment conducted by Jing in (2004). Spraying was done twice over a day on a regular basis for 25-30 days as requisite. Data was recorded repeatedly from 30 day's mature treated potatoes.

3.6. Preparation of media and different hormonal solutions

In order to culture meristem tips (Murashige and Skoog, MS) media was used as a fundamental medium and seven different hormonal applications were taken in consideration for meristem injection. Later than shoot renewal from callus initiated from meristem tips, subsequent sub culturing was done in three not similar 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 callus was also done with the finest treatment set up from testing 3 for dynamic and speedy multiplication of plantlet resultant from meristem culture.

3.7. Preparation of different stock solutions and MS media

The momentous feature for reaching of plant tissue culture was choice of suitable culture media with accurate concentrations of nutrient components and

growth regulator. For *in vitro* renaissance of potato MS medium (Murashige and Skoog, 1962) was used as basal medium. The table presenting masterpiece of MS medium is presented in Appendix-1. MS medium was used singly or with diverse concentrations of hormones in proportion to the requirements. MS media was prepared using stock solutions which was prepared and stored at 4℃ temperature well prior to the preparation of MS media.

3.7.1. Preparation of stock solutions

MS medium is equipped by the combination of stock solutions with diverse minerals and hormones requisite for plant growth improvement. Each stock solution composed of several types and amount of foremost salts, minor salts, iron and organic, growth regulators etc. correspondingly. All the chemicals used for stock solution is extremely purified and labeled as plant tissue culture tested mark. The chemicals are dissolved in double distilled water or greatly purified de-ionized water. Each chemical are added in proportion to the list of component existing in Appendix-I.

3.7.2. Preparation stock solution of macro nutrients (stock-I)

Stock solution of macro nutrients or stock-I was made ready with 10 times (10x) of the concluding strength of the medium in 1000 ml of distilled water. Salts are weighted precisely and liquefied entirely ten times the heaviness of the salts red for one liter of medium in 750 ml of distilled water. Every single chemical was dissolved fully earlier than adding other chemicals. The absolute volume was made up to one litre by the addition of distilled water. The stock solution was then filtered by using what man no. 1 filter paper to eradicate all the solid components. The stock I solution was amassed in a glass bottle, labeled with date of Preparation and stored in refrigerator at 4^0 C for upcoming use.

3.7.3. Preparation stock solution of micronutrients (stock-II)

The stock solution of micronutrients or stock-II was fabricated to hundred folds (100x) of the closing strength of the medium in 1000 ml of distilled water as

narrated previously same as stock I. Afterwards the stock solution was filtered as unchanged method as described before and tagged and stored in a refrigerator at 4^0C for further use.

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

The Stock-III solution is the kind of solution of iron-EDTA which was added just this minute and made up hundred folds (100x) of the final strength of the medium in 1000ml of solution. $FeSO_4$ and Na-EDTA were melted in 750ml of distilled water in a beaker by heating on a heater cum magnetic stirrer. The volume was completed up to 1000 ml by extra addition of distilled water. The preparation and storage of stock solution was finished in amber bottle or a bottle entirely covered with aluminum foil. The organized bottle was then stored in refrigerator at **4°**C.

3.7.5. Preparation of (stock-IV) solution

This stock solution contains vitamins and amino acids which is prepared by the addition of Pyridoxine HCI (Vitamins B_6), Thiamine HCI (Vitamins B_2), Myoinositol (Inositol), Glysine and Nicotinic acid (Vitamins B_3) individually. Each and every of the vitamins and amino acids except myoinositol were taken at 100 folds of (100x) to their concluding strength in a measuring cylinder and liquefied in 400 ml distilled water. Afterward the absolute volume was made up to 1000 ml by more addition of distilled water. At the end this stock solution was filtered and then stored in a refrigerator at 4^0 C for further use. However the myoinositol was prepared discretely 100 folds (100x) the absolute strength of the medium in 1000 ml of distilled water. This stock solution was as well filtered and stored in a refrigerator 4^0 C for later use.

3.7.6. Stock Solution for growth regulators

The previously prepared stock solution I, II, III and IV are requisite to prepare MS medium. There are some other dissimilar kinds of stock solution was equipped such as growth regulators (Hormones). Auxin and cytokinin are growth regulators

for mixing up into MS medium to sustain good growth of tissue, root, and shoot and other part enlargement according to the research prerequisite. In the present examination three types of growth regulators were used. They are 2,4-dichlorophenoxy acetic acid (2,4-D), Benzylaminopurine (BAP) and Gibberellic acid (GA3). Following chart shows the fine particles forms of growth regulators (solute) and their suitable solvent.

Table 2: Solubility percentage of some growth regulators:

Growth regulators *I* solute	Solvents
IBA (Auxin)	96% ethanol
2,4-D (Auxin)	96% ethanol
BAP(Cytokinin)	1 N NaOH
GA3 (Gibberellin)	Ethanol

***IBA = Indole-3-butyric acid, 2,4-D=2, 4-dichlorophenoxyacetic acid,**
BAP= Benzylaminopurine

On top of stated growth regulators were taken in containers in grind forms. To prepare growth regulator stock solution 50 mg of powder of every growth regulator were individually dissolved in their particular 50 ml solvents and then stored at 4 ± 1^0 C. Recently prepared growth regulators possibly will be used upper limit for two months. After the expired date fresh growth hormones were prepared for investigational use.

3.7.7. Preparation of other stock solutions

3.7.7. 1. 1 N NaOH preparation

40 gm of NaOH pellets were liquefied in one litre of distilled water to made up 1N NaOH. The prepared solution was then kept in chill and dried out place in a glass container. This solution was required for adjusting pH of ultimate MS

media preparation.

3.7.7.2. 70% Ethanol preparation

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

3.7.8. Preparation of MS media

One litre of MS medium was prepared according to the following steps narrated below:

- ✓ 100ml of stock Ⅰ, 10ml of stock Ⅱ, 10ml of stock Ⅲ and 10ml of stock Ⅳ were poured one after one in a one litre beaker which was located on a hot plate magnetic stirrer.
- ✓ 500ml of double distilled water was added into the beaker.
- ✓ 30 gm sucrose was added to the beaker and with the help of a magnetic stirrer sucrose was entirely dissolved gradually.
- ✓ Dissimilar concentrations of hormonal chemicals were added in accordance with the investigational supplies.
- ✓ The organized chemical combination was then poured in one litre of measuring cylinder and the volume was completed up to the level by the addition of double distilled water.
- ✓ Then the pH of the solution was attuned to 5.8 using a pH meter. For amendment of the pH 1N NaOH and 0.1N HCI were used as per necessity.
- ✓ 8 gm Agar was added to the combination and heated in a micro wave oven at 100℃ gradually until the agar dissolves wholly.
- ✓ The MS medium is now prepared to sterilize and after sterilization it is organized to use.

In order to sterilization the warm MS medium is dispensed into culture vessels (of about 20m *medial* Vessel) or test tubes (of about 10ml medial test tube). After allotment of media the test tube was wrapped with aluminum foil and each test tube was labeled for hormonal combinations with the help of a glass marker pen.

3.8. Sterilization steps

3.8.1. Sterilization of culture media

The culture vessels or test tubes with just now equipped culture media were sterilized under 15 psi at 121^{o}C for 20 minutes in an autoclave apparatus. The medium was chilled at room temperature previous to use.

3.8.2. Sterilization of glassware and instruments

All types of glassware, measuring cylinder, test tubes, culture vessels etc and all the metal instruments were cleaned with liquid detergent and washed with running tap water and then washed again with distilled water. The cleaned glassware's were autoclaved under 15 psi at 121℃ for 30 minutes. After wards all the metal instruments which are to be used in laminar air flow during culturing and outer surface of laminar air flow during operating a diverse of works in media preparation, chemical measurement etc later than washing were wrapped with aluminum foil and autoclaved under 15 psi at 121℃ for 30 minutes.

3.8.3. Sterilization of culture room and transfer areas

Prior to use of culture room it was cautiously cleaned with detergent and wiped with 70% ethanol. The room was ssterilized with formaldehyde. Great safety measures was taken during formaldehyde spraying such as, protective mask was put on, whole body was covered with protective cloths, wearied the gloves, protective glasses etc to save the skin from any harm cause by this chemical. Subsequent sterilization of the whole room it was concealed for 24 hours. After that entering the room all the rakes and shelves were exterior

cleaned with 70% ethanol. This practice was applied at regular intervals.

3.8.4. Sterilization of laminar air flow cabinet

The laminar Air flow cabinet was in progress for thirty minutes ahead of working and sterilized fine in advance. The Air flow cabinet exterior part was cleaned with yarn soaked with 70% ethanol. The lead of cabinet then clogged finely and UV was switched on while turning off the air flow. The UV light of cabinet was left on for half an hour. After UV sterilization the surface area of the laminar Air flow was wiped up and clean up with 70% ethanol. In a view not to rising the occurrence of contamination aluminum foil rolled up pre-sterilized all equipments which was used for the period of culturing reserved in airflow cabinet switching on the UV light for an additional 10-15 minutes just prior to starting culture. During the culture all the equipments were repeatedly flame sterilized after plummeting into 70% recently made ethanol. Hands were washed accurately and sterilized with 70% ethanol.

3.9. Environment of culture room

A controlled environment was organized in order to keep the Meristem cultured and sub cultured vessels and test tubes. The temperature of culture room was maintained inside 25±1℃ with the assist of air conditioner. The cultured room environment was maintained with 16 hours Photoperiod and the aid of white florescent lights by using 100W bulbs. The light intensity was controlled 3000 lux and monitored by using lux meter.

3.10. Protocol of meristem culture

3.10.1. Collection of Explants

In order to collection of explants, potato tubers were treated with GA3 (400ppm/l) and left for sprouting. According to Stone (1963) only shoot tips between 0.2 and 0.5 mm most frequently produce virus free plantlets. So as soon as a sprout reaches about 0.5 - 1 cm in length, meristem was collected from sprouts for culturing purpose. Meristem was the preliminary substance for

culture.

3.10.2. Surface sterilization and meristem culture

Potato sprouts were sheared with the help of sterilized surgical blade and collected into six unlike 50ml beaker to receive six varieties which was labeled well with marker pen. Sprouts were cleaned with distilled water twice. Then washed sprouts were taken under laminar air flow which has been sterilized ahead of starting culture. For surface sterilization, sprouts were yet again washed with sterilized double distilled water. After that potato sprouts were immerged with 70% ethanol for 1 minute then washed three times gently with double distilled water. After washing, sprouts were dipped into 0.1 % $HgCI_2$ solution additionally 3 drops of Tween-20 was poured and kept for 4 minutes. After that sprouts were washed again with double distilled water four times to eliminate any type of chemicals and dirt's that remains on sprouts. After cautiously removing further water by using sterile blotter paper, sprouts were placed on a clean sterilized Petri dish.

3.10.3. Meristem dissection

Meristem was taken from potato shoot tip or sprouts. Shoot tips were enclosed by various soft leaf primordia. Under 100X magnification binocular microscope the external leaf primordial which surrounding the meristematic region was redundant with the help of a sharp microsurgical blades and pointed fine needle, awaiting only the meristematic region and one or two leaf primordia about 0.5 mm leftovers intact (Figure 2). Special concern was taken not to injure the tissue at the time of dissection of meristem as this part was the most significant and delicate part in the conduct experiment.

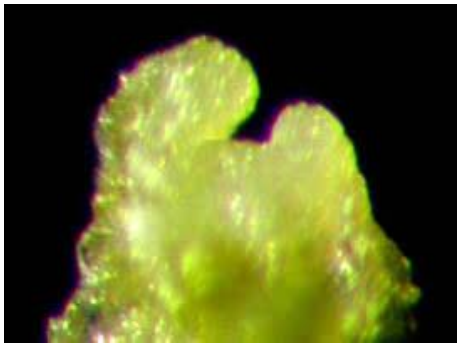


Figure 2. Potato tuber meristem seen under the stereomicroscope

3.10.4. Inoculation of meristem

One by one meristem tip of the sprouts were dissected and keeping the cut surface touching with the media. Extraordinary care was taken to keep the particularly minute sized cut pieces of meristem integral and undamaged. After inoculation each test tube was without delay wrapped with aluminum foil. The cultured test tubes were placed in rakes in restricted environment.

3.11. Observation and data collection

Cultured meristem was frequently executed to detect any observable change of growth and any color improvement. Unhygienic or dead cells or impassive materials were removed and data was recorded. Each treatment was done with 4 replications and survival rate was also recorded. Three randomly selected replications from every treatment were chosen for data collection.

- Survival Percentage of explants inoculated (%)
- Callus texture and color
- Days to callus initiation
- Callus size measurement (length and breadth in cm) at 28 days

After callus initiation and rejuvenation of particular shoot on the same culture, Data was also taken on standard basis on following parameters.

- ✓ Days to shoot initiation
- ✓ Shoot length/callus (at 7,14, 21 and 28 DAI)
- ✓ Days to root initiation

3.11.1. Subculture of single shoot

Primarily sub culturing was done using distinct shoot (28 DAI) induced from callus in meristem culture to redevelop more shoots where three levels of several growth hormones were used. 21 days after subculture following data was taken on regular basis,

Number of leaves/ plantlet
Number of roots/ plantlet
Root length/plantlet

3.11.2. Subsequent sub culturing

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

Number of roots/plantlet

Root length/plantlet (Maximum length in cm)

Number of leaves/plantlet

3.11.3. Continuous sub culture of shoot cuttings

After taking on top of cited data continuous sub culturing was done to obtain vigorous plantlets using 2 cm long shoot cutting. Later than doing 3-4 sub culturing all the plants displayed vigorous growth at soaring light intensity.

3.11.4. Treatments

Three experiments were carried out to evaluate the outcome of sprouting abilities of potato, the effect of diverse concentration of IBA, 2, 4-D, and BAP on callus induction, shoot rejuvenation and plantlet establishment.

3.12. List of experiments and Data collection methods

3.12.1. Experiment 1: Effect of GA3 treatment on sprouting abilities of three potato cultivars.

Design: CRD

Replication: 3

Explant: Potato tuber

Number of potato used per treatment: 3

Treatment details: GA3 400ppm/L

3.12.1.1. Days required to initiate sprouting

During regular spraying of potato it was cautiously noticed to see any germination of sprouts. Beginning date of spraying was filed for each treatment and every variety. As soon any observable symbol of sprouting was seen on that day data was recorded and number of days was counted to locate the number of days requisite to initiate sprouting.

3.12.1.2. Number of sprout / potato

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

3.12.1.3. 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.

3.12.1.4. Maximum sprout length (cm)

Highest sprout length in centimeters was taken and filed from 30 days matured potatoes treated with diverse concentrations of GA3.

3.12.2. Experiment 2: Effect of 2,4-D, BAP and IBA, in callus induction , shoot and root regeneration from meristem tip culture, of selected three potato cultivars

Design: CRD

Replications: 4

Explant: Meristem / shoot tip from potato sprouts

Number of explant inoculated per replication: 1

Total treatment: 7 (including control)

In this experiment, the effect of diverse concentration and combination of 2, 4-D, BAP and IBA were used with MS media to detect the best combination for meristem culture. In these experiment six different combinations of 2, 4-D, BAP and IBA were used and a control treatment without hormonal combination was also taken into consideration (Table 3).

Table 3: Different types of hormonal combination:

Hormonal Treatment	IBA + 2, 4-D + BAP(mg/L)
T_1 (Control/Normal MS)	0+0+0
T_2	0.25+0.125+0.5
T_3	0.25+0.25+1.0
T_4	0.25+0.37+1.5
T_5	0.25+0.50+2.0
T_6	1.50+1.0+0.5
T_7	1.50+2.0+1.0

***IBA=Indole-3-butyric acid, 2,4-D= 2, 4-dichlorophenoxyacetic acid, BAP = Benzylaminopurine**

3.12.2.1. Survival rate

The survival rate of injected meristems were recorded. In the beginning there are four replications of each treatment of each variety were prepared. Number of explants maintained to survive and were recorded by using following formula:

$$\text{Survival rate (\%)} = \frac{\text{Number of established plantlets}}{\text{Total number of plantlets}} \times 100$$

3.12.2.2. Days to callus initiation

Recently cultured meristems were noticed every day. While any alteration of meristem or development of callus in cultured explants were executed and data were recorded as days to meristem spouting or days to callus commencement.

3.12.2.3. Callus color

30 days matured meristem culture was observed for studying the callus culture. In some cases callus was formed. Color of callus was recorded.

3.12.2.4. Callus texture

Callus are emerged under diverse treatments of 30 days matured cultures were cautiously observed for their substantial characteristics (friable and non friable) and data was recorded.

3.12.2.5. Callus Size

Length and breadth of 30 days matured callus were calculated in centimeter (cm) by using a measuring scale. Size of the callus was recorded at 7, 14, 21 and 28 DAI after callus insertion. Length of the callus was measured vertically and breadth was measured horizontally. The following formula was used for calculating the size of the callus is given below(Thadavong *et al*. (2002):

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

3.12.2.6. Single shoot callus

Single shoot enlargement occurred following development of callus from meristem to some amount. These cultures were carefully executed for shoot commencement and rejuvenation. Data was recorded for instance days to shoot initiation in these cases. When single shoot was appeared date was recorded to find out at days requisite to initiation of shoot.

3.12.2.7. Shoot length/plantlet

Shoot length of cultured explants was recorded 7, 14, 21 and 28 DAI.

3.12.2.8. Number of shoot/plantlet

Number of shoot that formed from the explants were counted and data was recorded from 21 days mature subcultures and mean data was calculated.

3.12.2.9. Number of leaves/ plantlet

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

3.12.2.10. Days to root initiation

The meristem cultures were gingerly noticed each day for root advancement and when any root development and elongation was seen that was treated as days to root initiation.

3.12.2.11. Root length in centimeters

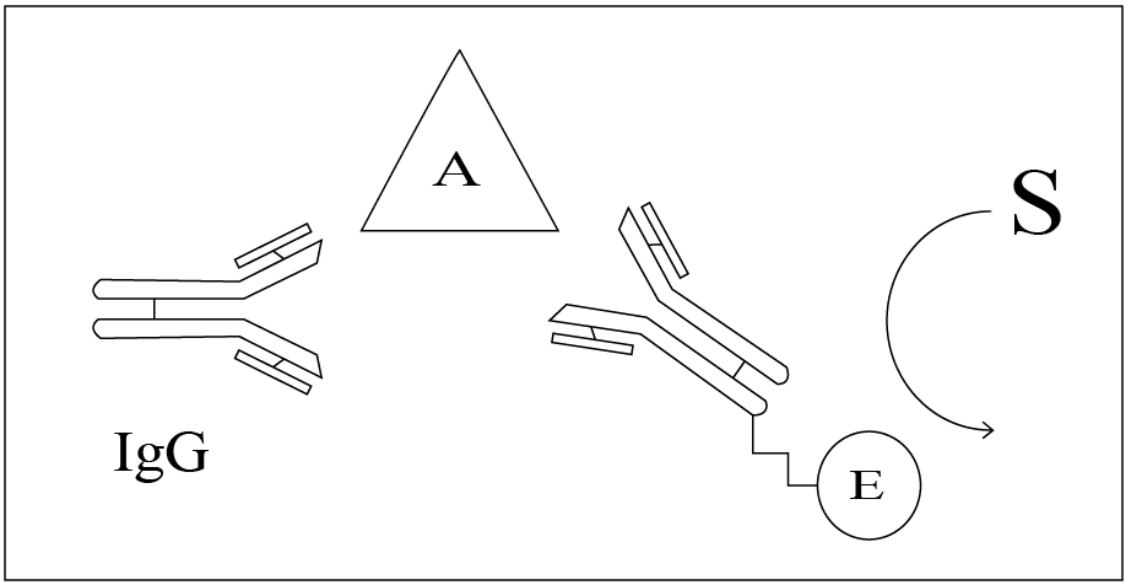
Root length was measured in centimeter (cm) from bottom to the angle of the roots from 21 days old subcultures and average length of the roots was recorded and mean was premeditated.

3.13. Experiment 3: ELISA test

Primary plant materials were in the beginning reported virus infected during carrying out experiments (Alam, 2011) in field conditions throughout that time.

Virus infected plants were observed in field environment and photo was taken (Plate 2). Afterwards completion of the meristem culture experiment effectively regenerated plantlet samples of each variety was taken to the tissue culture laboratory of BCSIR (Bangladesh Council of Scientific and Industrial Research, Dhaka) for ELISA (Enzyme linked Immunosorbent Assay) test using DAS-ELISA protocol (BARI) to authenticate that the plantlets obtained from meristem culture were virus free.

3.13.1. ELISA kit collection



ELISA kits were collected from the local supplier. The brand name of the ELISA chemicals was “Life Science” and “Agdia”. DAS-ELISA test was performed to detect the PLRV, Potato Virus A, Y, X, M, S, V. The Double Antibody Sandwich ELISA (DAS) uses antibodies (IgG) which are bound to the surface of a microtitre plate to capture the antigen (A) of interest. A specific antibody enzyme conjugate (E) is then used to detect the trapped antigen. The presence of enzyme (in this case alkaline phosphate) is detected by a colorimetric substrate (S) reaction.

3.13.2. Reagents required for DAS-ELISA test

Coating antibody (codes in the ADGEN catalogue ending in -01/-02

Conjugate (ADGEN codes ending in -03/-04)

Coating buffer (ADGEN codes 02-001/02-002)

Phosphate buffered saline + Tween 20 (ADGEN code 02-003)

Extraction Buffer (ADGEN codes 02-004 - 02-016 depending on antigen of interest)

Conjugate buffer (ADGEN codes 02-008/02-009)

pNNP tablets (ADGEN code 0-001/0-002)

Substrate buffer (ADGEN code 02-010/02-011)

Alternatively for your convenience ADGEN supply a DAS ELISA buffer pack (02-017/02-018) and prepared liquid substrates which are stable, convenient and easy to use (03-003/03-004) and for enhanced detection in your assay choose ADGEN blue liquid substrate system (03-005/03-006)

Additionally positive and negative controls are available from ADGEN (-11/-12) for a wide variety of pathogens, these allow you to have increased confidence in the performance of the assay.

To cover all of your ELISA requirements ADGEN supply ADGEN total sets which contain all of the antibodies, reagents and controls that are required for testing. ADGEN Total is available in 500, 1000, 2500 and 5000 unit sizes.

3.13.3. Recommended buffer for DAS-ELISA

3.13.3.1. Coating Buffer (Carbonate buffer)

Sodium carbonate	1.59g
Sodium hydrogen carbonate	2.93g

Make up to 1 litre with dH_2O. The pH of this buffer is 9.6 and does not require to be adjusted.

3.13.3.2. Phosphate buffer saline (PBS) ×10

Sodium chloride	80g
Potassium diHydrogen orthophosphate	2g
Disodium Hydrogen orthophosphate	11.5g
Potassium chloride	2g

Make up to 1litre with dH_2O. The pH of this solution when diluted to 1×s is 7.2

3.13.3.3. Wash buffer (PBS+Tween 20)

Phosphate Buffer Saline	1 litre
Tween 20	0.5ml

3.13.3.4. Grneral Etraction Buffer

Polyvinylpyrrolidone (PVV)	20g
Ovalbumin	2g
Sodium sulphite (anhydrous)	1.3g
Sodium azide	0.2g
Tween 20	0.5ml
Sodium Chloride	8g
Potassium diHydrogen orthophosphate	0.2g
Disodium Hydrogen orthophosphate	1.15g
Potassium chloride	0.2g

Make p to 1 litre with distilled/deionised water. This buffer can be difficult to get into solution and it is easier if the PVP is mixed into a "paste" with a small volume of water before adding the other components and the remainder of water.

3.13.3.5. Conjugate buffer

Bovine serum albumin	0.2g
PBST	0.2g

3.13.3.6. Substrate buffer (Diethanolamine buffer 1M)

Dietanolamine	90.39g
Dietanolamine -HCl	19.82g
Magnesium chloride	0.1g

Make up to 1litre with dH_2O. The pH of the buffer is 9.8 and it does not require to be adjusted. (The diethanolamine and dietanolamine-HCl are liquids however; it is easier to weight them out than to measure their volumes as they are extremely viscous.)

pNPP is added to the above buffer at 1mg/ml to make up the substrate for alkaline phosphatase.

3.13.3.7. Protocol

1. Dilute coating antibody in coating buffer as recommended on the bottle label and add 100µl to the required number of wells for the test.

2. Wrap the plate tightly in cling film or place in a plastic box with some damp paper towels and close the box. Incubate the plate at 37^0C for 4 hours.

3. Wash the plate three times with phosphate buffered saline + Tween 20 (0.05) –PBST. To do this fill the wells of the plate with PBST and invert to remove the buffer. Repeat twice, pat the plate dry on paper towels.

4. Extract the samples by grinding 1g of tissue with10ml of general extraction buffer in a mortar and pestle for an alternative method of grinding. Then filter the sample through a layer of muslin (or similar fine cotton gauze). If this is not available then allow the plant material settle and use the supernatant in the test. In some cases the recommended ratio of sample to buffer may have to be reduced to allow a clear signal to be obtained if the plant material is not highly infected.

5. Add 100µl of each sample, positive and negative control to the coated wells. ADGEN recommended that all samples and controls are tested in duplicate. Remember, 1 ADGEN UNIT= 2 TEST WELLS.

ADGEN positive and negative controls are reconstituted by adding 2ml of distilled/deionised water and gently shaking. Any unused reconstituted control may be stored at -20^0C. However, the performance of the positive controls may decrease when stored in this manner.

6. Wrap the plate as described in (2) above and incubate at 4^0C overnight (at least 16 hours).

7. Wash the plate as described in (3) above.

8. Dilute the antibody-enzyme conjugate as recommended on the bottle label in conjugate buffer and add 100µl to each test well.

9. Wrap as in (2) above and incubate at 37^0C for 1hour.

10. Wash four times as described in (3) above. An extra wash is included at this stage to ensure that all unbound antibody-enzyme conjugate is removed from the wells.

11. Prepare the substrate just before use – add pNPP at 1mg/ml to substrate buffer (one 5g tablet in 5ml of buffer). Alternatively use one of the ADGEN liquid substrates. All of these substrates may change color when exposed to light and should be protected from light to prevent this occurring.

12. Add 100µl of prepared substrate to each test well.

13. Wrap the plate as in (2) above and incubate in the dark ar room temperature for 1 hour.

14. Read the absorbance using a spectrophotometer at 405nm (for pNPP and ADGEN Yellow) or 595-650nm (for ADGEN Blue). Alternatively positive and negative samples may be scored visually although this may not be as accurate as using a spectrophotometer. A positive sample may be determined as one which gives an absorbance value which is greater than the absorbance values of the negative control. A negative sample is one which gives an absorbance value which is the same as, or less than the negative control. Visually a positive sample will give adarker color than the negative control and a negative sample will give a similar or lighter color to the negative control.

3.14. Acclimatization

Well developed plantlet with developed root was withdrawn from the culture vessels very cautiously avoiding the damage of the roots. Any form of culture medium was washed away from the roots with running tap water. Then the roots were washed with distilled water. After that 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.

3.15. Transfer of plantlets to soil

After adaptation of plantlets they were shifted to net house, where proper care was taken for expansion and development of potato plantlet. Later than 20 days matured plantlets exhibit the highest vigorous growth and was transferred to the field in normal field condition.

3.16. Experimental design and statistical Analysis of Data

All the trials such as meristem culture, callus instigation, plant regeneration, and subculture were done under restricted laboratory condition, which was supposed to be homogeneous in such condition. As such, the research was carried out in the completely randomized design (CRD). The collected data on different parameters were analyzed using a STATISTIC-10 package computer program. The analysis of variance was completed and means were compared by Least Significant Difference (LSD) test for explanation of results.

CHAPTER 4

RESULTS AND DISCUSSION

The present experiment was carried out to develop the reproduction protocol of virus free potato mini-tuber with the intention to supply virus and disease free planting materials. The main purpose of this study was to produce in vast quantities for large scale cultivation from regeneration of potato plantlets which was obtained from meristem culture. *In vitro* regeneration of three potato cultivars by using different combinations of 2, 4-D BAP and IBA on meristem culture, shoot and root regeration were studied. The results were gathered from this experiment have been presented and discussed under following headings and sub – headings.

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

Collection of meristem is one of the major task in meristem culture procedure. In this trial meristem was culled from potato sprouts (Figure 3). Present experiment was carried out to get highest sprouts moderately in short periods of time. Two levels of solutions concentrated with GA3 (0.0 and 400 ppm) was sprayed to three potato varieties. Results have been presented in (Table 4) a range of treatments displayed considerable influence during the research over days essential to commence sprouting, number of sprouts/ potato, maximum sprout length, and number of sprouts/eye. Results are discussed under following heading.

4.1.1. Experiment 1: Effect of GA3 treatment on sprouting abilities of selected three potato cultivars

4.1.2. Days required to initiate sprouting

Selected potato cultivars viz. Granola, Diamant and Cardinal potato tubers taken longer period of time for sprouting (9, 11 and 10 days required to initiate sprouting respectively) to respond when treated with 400 ppm. Whereas Granola, Diamant and Cardinal responded early when treated with 400 pm GA3 (3, 5 and 4 days required to initiate sprouting respectively). Among the three selected potato cultivars Diamant responded very slowly (5) days required to initiate sprouting) at 400 ppm GA3 application whereas Granola took shortest feasible time (3 days) to induce sprouting while they were treated with 400 ppm GA3 solutions.

Table 4: Sprouting potentiality of three BARI released Potato tubers under different concentrations of GA3 treatment

GA3 Treatment (ppm/L)	Variety	Days to sprout initiation	Number of sprout /potato	Number of sprouts/eye	Length of sprout (cm)
0	Granola	Sprouting was not occurred in off-season			
	Diamant				
	Cardinal				
400	Granola	3	6	4	1-1.6
	Diamant	5	5	3	3-3.5
	Cardinal	4	5	5	4-4.2

4.1.3. Number of sprouts/potato

Number of sprouts/potato was maximum (6) by the cultivar Granola with the application of GA3 at the rate of 400 ppm which was advantageous in this test

to get utmost number of sprouts relatively in short period of time. While Diamond and Cardinal produced less (5) amount of sprout per potato at the mentioned concentration (Table 4).

4.1.4. Number of sprouts/eye

Number of sprouts/ eye was observed maximum (5) when GA_3 was applied at 400 ppm concentration, and minimum (3) number of sprout was produced by Diamant cultivar. Variety Granola produced (4) number of sprouts at the mentioned GA_3 concentration.

4.1.5. Maximum sprout length (in Centimeter)

Maximum length of sprout was recorded in Cardinal (4-4.2 cm) under 400 ppm GA3 application and minimum sprout length was recorded in Granola (1-1.8cm) under 400 ppm GA3 application. Results have been displayed in (Table 4). Almost all three potato varieties showed noteworthy results in response to 400 ppm GA3 application in respect of days required to initiate sprouting. 400 ppm GA3 treatment was chosen as finest treatment and used to get highest sprouts for the compilation of meristematic shoot tip in shortest feasible time.

A

B

C

Figure 3. Maximum sprouting at GA_3, 400 ppm/l concentration, Sprouts of Granola (A), Diamant (B) and Cardinal (C)

4.2. Experiment 2: Callus induction and plant regeneration of three potato varieties supplemented with diverse concentration of IBA, 2,4-D and BAP

4.2.1. Callus Texture and Callus Color

The cut edge of meristem initially a creamy white appearance occurred. Gradually they developed different colors in different potato cultivars calli were mostly changed into green, yellowish green, pink or whitish green (Table 5). At 28 days after inoculation of meristem, cultured meristem formed calli which was examined for determining texture of callus. The callus texture was friable/ non friable.

Table 5: Texture and color of callus of three potato genotypes at 28 DAI

Variety	Callus color	Callus texture
Granola	Yellowish green	Friable & loose
Diamant	Greenish	Friable& loose
Cardinal	Whitish green	compact

4.2.2. Days to Callus induction

Days to callus initiation was recorded after inoculation of explants at 28 days of cultured on MS media containing diverse concentrations of IBA, 2,4-D and BAP. The major effect of all three varieties showed that there was a significant difference showed on days to callus initiation among the three varieties the . Maximum days (7.59 days) were required for callus initiation on the variety Cardinal and the minimum days (3.62 days) were required for callus initiation in the variety Granola

In case of Diamant variety the callus initiation and proliferation time was (5.13 days). Results have been presented in (Table 6) and (Figure 4).

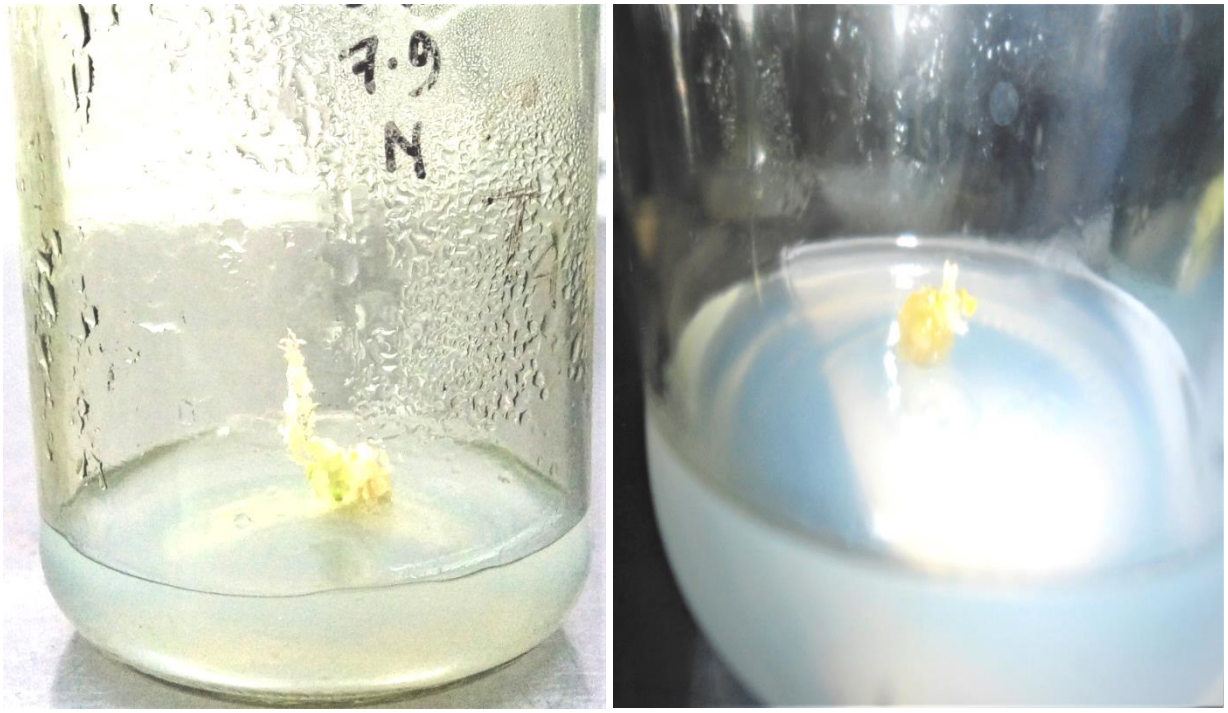
The three potato cultivars and diverse levels of hormonal treatments revealed

significant interface in relation to the number of days to callus initiation (Table 7). The maximum days (9.0 days) required for callus initiation was noticed in Diamant with the hormonal combination T_7 (1.50 mg/l IBA +2.0 mg/l 2,4-D +1.0 mg/l BAP). The minimum days (2.0 days) required for callus initiation was observed in Granola when cultured in T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP). No callus formation occurred to all the varieties when cultured in normal MS media.

4.2.3. Size of callus

The size of callus of the three studied potato varieties were recorded cultured on MS media supplemented with different hormonal combinations of IBA, 2,4-D and BAP and showed significant difference among them. There was a significant difference in size of callus among these potato cultivars (Table-6). Maximum size of callus (0.61 cm) was recorded in Granola whereas minimum callus size (0.27 cm) was recorded in variety Diamant.

The various hormonal treatments displayed significant variations on callus size while explants were inoculated in different hormonal treatments owing to interaction effect of dissimilar potato varieties and hormonal treatments on size of callus (Table 7). The uppermost size of callus (0.74 cm) was found in Granola when inoculated in concentration T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) whereas minimum size of callus (0.18 cm) was recorded in Diamant when inoculated in T_2 (00.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP). Combination no callus was formed in T_1 (fresh MS media) in case of all varieties.



A B

C

Figure 4. Initiated callus after inoculation at 2-3 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2, 4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

Table 6: The Effect of three potato cultivars on days to callus initiation and proliferation:

Potato genotypes	Days to callus initiation	Callus Size (cm)
Granola	3.62	0.61
Diamant	5.13	0.27
Cardinal	7.59	0.30
LSD	0.75	0.04
Level of significance	**	**

Table 7: Combined effect of different varieties and different hormones on Callus initiation and number of shoots/plantlets:

	Treatment	Days of callus initiation	Callus size
Granola	T_1=Normal MS	-	-
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	6.00	0.24
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	2.00	0.74
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	3.00	0.58
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	8.00	0.60
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	7.00	0.25
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	7.00	0.36
Diamant	T_1=Normal MS	-	-
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	7.00	0.18
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	4.00	0.44
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	4.00	0.30
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	3.00	0.35
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	3.00	0.21
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	9.00	0.20
Cardinal	T_1=Normal MS	-	-
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	7.00	0.36
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	3.00	0.63
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	5.00	0.69
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	5.00	0.66
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	5.00	0.56
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	6.00	0.63
	Max	9.00	0.74
	Min	2.00	0.18
	LSD	1.88	0.11
	Level of significance	**	**

4.2.4. Days to shoot initiation

Days to shoot initiation was recorded after 7, 14, 21 and 28 days of cultured on MS media containing different concentrations of 2,4-D, IBA and BAP. Results have been presented in (Table 8-9) and (Figure 5). All the three varieties were differed in days to shoot initiation. Maximum days to shoot initiation was recorded in variety Cardinal (14.88 days) (Table 8). Ao and Liu (1998) observed the varietal differences of shoot regeneration in potato.

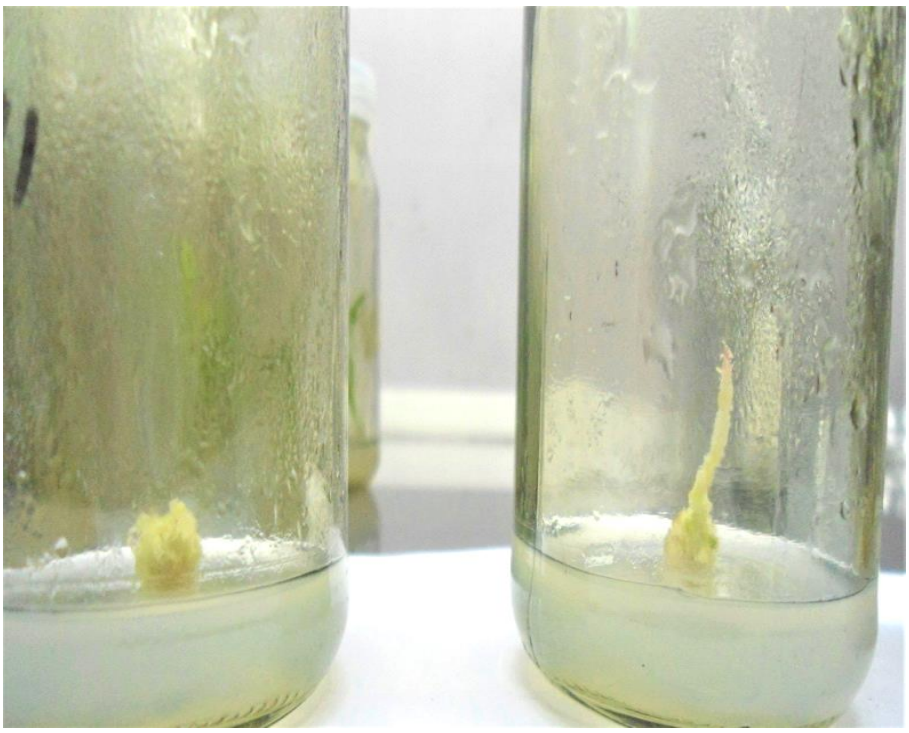
Table 8: Effect of different varieties on days to shoot initiation and number of shoots/plantlets at different days after inoculation

Variety	Days to Shoot initiation	Number of shoots/plants			
		7 DAI	14 DAI	21 DAI	28 DAI
Granola	13.15	0.84	1.59	2.13	3.02
Diamant	13.76	0.52	0.92	1.52	2.12
Cardinal	14.88	0.44	0.91	1.44	1.97
CV	12	26	20	16	13
LSD	0.995	0.128	0.198	0.217	0.365

Combined effect of different treatment and potato genotypes on days required for shoot initiation (Table-9) revealed that Diamant took maximum days to initiate shoot (21.36 days) when cultured in media containing T_7 (1.50 mg/l IBA +2.0 mg/l 2,4-D +1.0 mg/l BAP) whereas Granola and Cardinal took minimum days (5.34 days and 7.64 days))for shoot initiation when cultured in T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) and Diamant showed no response for shoot initiation when cultured in media containing T_5 treatment (0.25 mg/l IBA +0.50 mg/l 2,4-D +2.0 mg/l BAP).

Table 9: Combined effect of different varieties and different hormones on number of shoots/plantlets

	Treatment	Number of shoots/plantlets			
		7 DAI	14 DAI	21 DAI	28 DAI
Granola	T_1=Normal MS	1.00 a	1.33 ab	1.67 bc	2.67 bc
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	1.67 a	2.34 a	3.00 a	3.33 ab
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	1.36 a	2.00 ab	3.33 a	4.33 a
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	1.33 a	1.67 ab	2.34 abc	2.67 bc
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	1.32 a	2.33 a	2.68 ab	3.68 ab
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	0.00 b	1.00 b	2.33 abc	3.32 ab
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	0.00 b	1.00 b	1.33 c	2.00 c
Diamant	T_1=Normal MS	1.00 b	1.00 c	1.00 c	1.67 c
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	1.39 ab	1.67 b	2.00 ab	2.69 b
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	1.33 ab	2.00 ab	2.33 a	3.33 ab
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	1.67 a	2.33 a	2.67 a	3.67 a
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	0.00 c	0.00 d	0.00 d	0.00 d
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	0.00 c	0.00 d	1.33 bc	1.33 c
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	0.00 c	0.00 d	1.33 bc	1.37 c
Cardinal	T_1=Normal MS	1.00 a	1.33 b	1.67 cd	3.62 ab
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	1.33 a	2.00 a	2.33 bc	2.67 c
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	1.37 a	1.67 ab	3.33 a	4.00 a
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	1.32 a	2.00 a	2.67 ab	3.00 bc
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	1.34 a	1.68 ab	2.62 ab	3.00 bc
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	0.00 b	0.00 c	1.00 d	1.33 d
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	0.00 b	0.00 c	1.00 d	1.33 d
	Max	1.67	2.34	3.33	4.33
	Min	1.00	1.33	1.00	1.33
	CV	73	58	39	42
	LSD	0.76	0.66	0.85	1.01



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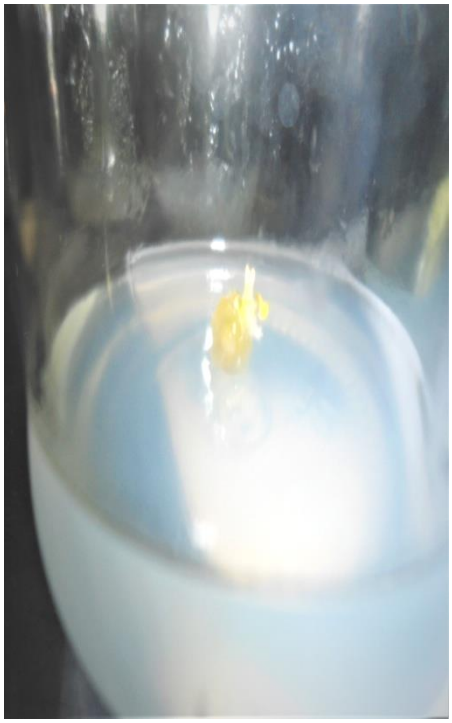


C

Figure 5. Initiated shoot at 7 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

In case of Granola, shoot was formed in MS media within 7.34 days. With the application atT_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP) combination, Granola shoot was induced at (5.67 days), whereas at T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) combination revealed the minimum percentage (5.34 days) of shoot initiation as compared to other treatments and other varieties. Variety Granola induced shoot at T_4 (0.25 mg/l IBA +0.37 mg/l 2,4-D +1.5 mg/l BAP) combination and it took 6.67 days, followed by 9.34 days, 12.34 days and 14.35 days at T_5 (0.25 mg/l IBA +0.50 mg/l 2,4-D +2.0 mg/l BAP), T_6 (1.50 mg/l IBA +1.0 mg/l 2,4-D +0.5 mg/l BAP) and T_7 (1.50 mg/l IBA +2.0 mg/l 2,4-D +1.0 mg/l BAP) concentration. Results are presented in (Figure 6).

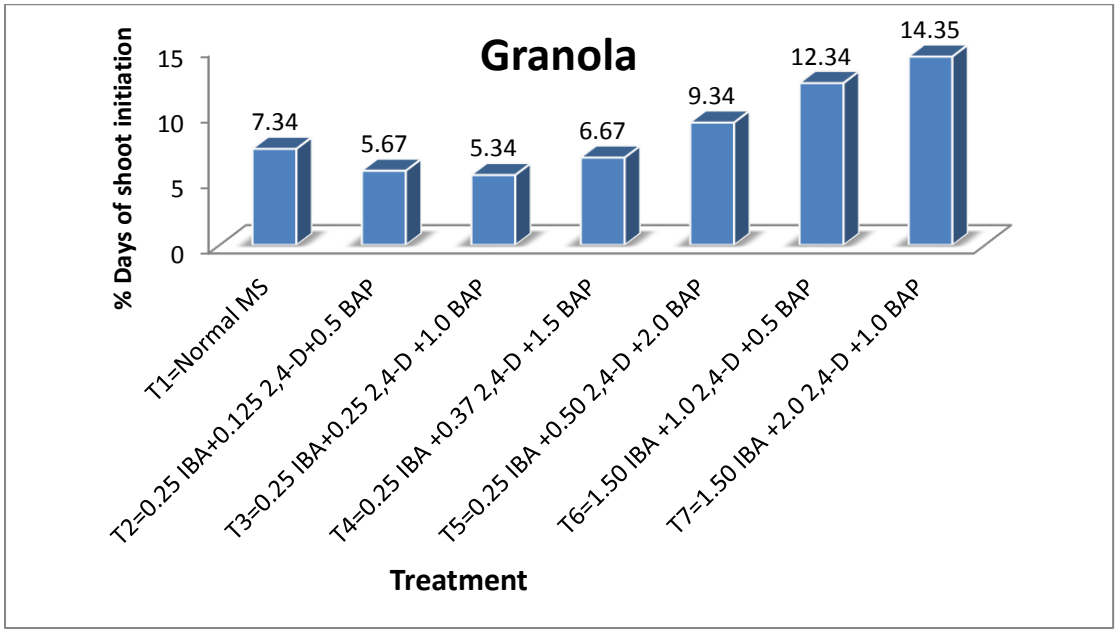


Figure 6. Effect of different hormones on percentage of days to shoot initiation of Granola

In case of Diamant, shoot was formed in MS media within 7.0 days. With the application of T_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP) combination. It was formed within 7.31 days and at T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) combination, it was 7.64 days T_4(0.25 mg/l IBA +0.37 mg/l 2,4-D +1.5 mg/l BAP) shoot induction was revealed the minimum percentage at (6.68 days) of shoot initiation as compared to other treatments. At T_5 (0.25 mg/l IBA +0.50 mg/l 2,4-D +2.0 mg/l BAP) combination no shoot was induced in Diamant variety. Diamant took about 20.39 days and 21.62 days to induce shoot at a combination of hormone T_6(1.50 mg/l IBA +1.0 mg/l 2,4-D +0.5 mg/l BAP) and T_7(1.50 mg/l IBA +2.0 mg/l 2,4-D +1.0 mg/l BAP) combination, achieving the lower grade in case of days to shoot initiation. Results are presented in (Figure 7).

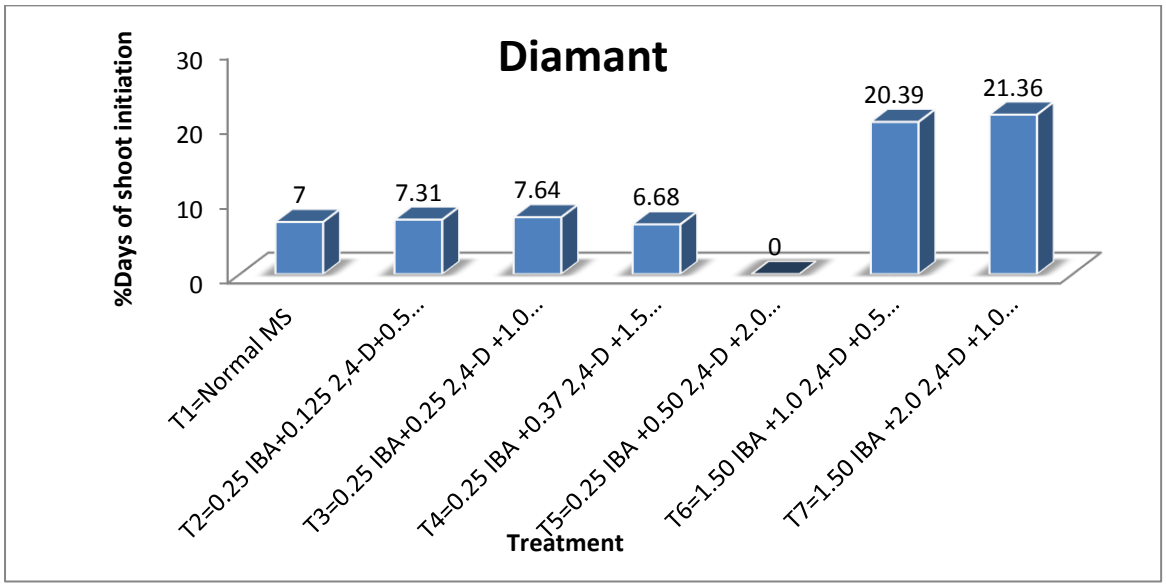


Figure 7. Effect of different hormones on percentage of days to shoot initiation of Diamant

In case of Cardinal shoot was formed in T_1 (MS) media within 8.67days. With the application of T_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP) combination, Cardinal shoot was induced at 9.33 days, whereas at T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) combination revealed the maximum percentage (7.64 days) of shoot initiation as compared to other treatments within less time. Shoot was induced within 9.35 days at T_4 (0.25 mg/l IBA +0.37 mg/l 2,4-D +1.5 mg/l BAP) combination and at T_5 (0.25 mg/l IBA +0.50 mg/l 2,4-D +2.0 mg/l BAP) combination it took 9.68 days. At T_6 (1.50 mg/l IBA +1.0 mg/l 2,4-D +0.5 mg/l BAP) and T_7 (1.50 mg/l IBA +2.0 mg/l 2,4-D +1.0 mg/l BAP) combination, it was required 21.33 days and 20.62 days for shoot induction. Results are presented in (Figure 8)

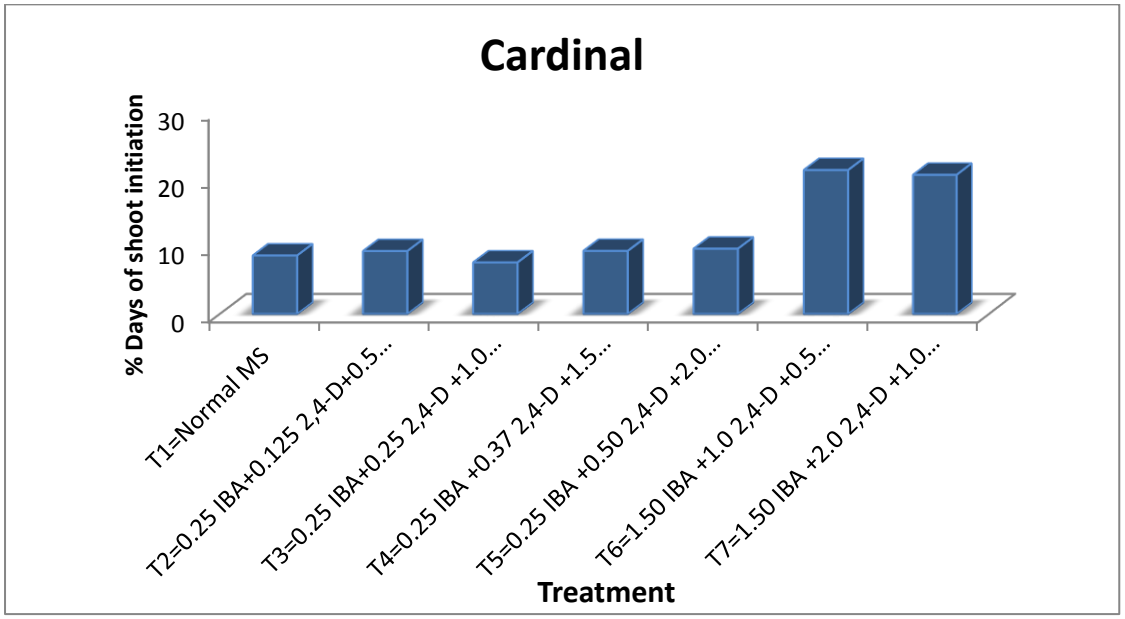
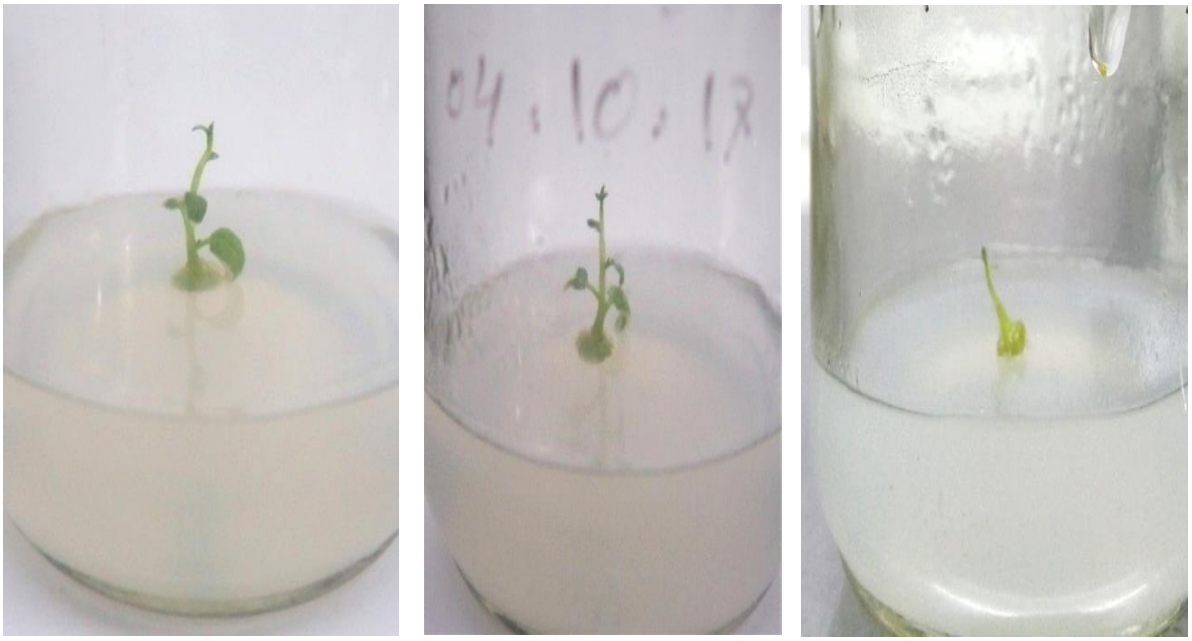


Figure 8. Effect of different hormones on percentage of days to shoot initiation of Cardinal

4.2.5. Number of shoots/plantlets

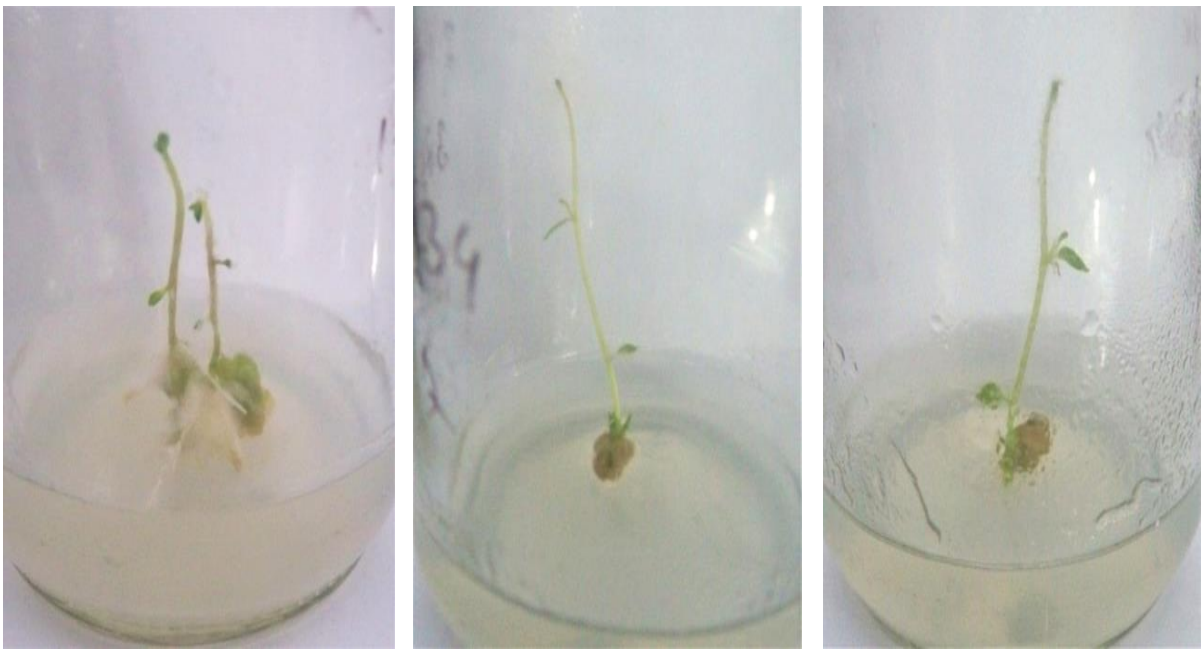
Number of shoots/plantlet under in vitro condition differed significantly among varieties (Table 8). The highest shoots/plantlet was recorded in Granola (0.84, 1.59, 2.13 and 3.02 shoots/plantlets at 7, 14, 21 and 28 DAI), while the minimum number of shoots/plantlet (0.44, 0.91, 1.44 and 1.97 shoots/plantlets at 7, 14, 21 and 28 DAI respectively) was found in Cardinal.

Combined effect of different treatment at different concentrations of hormones displayed most significant difference at distinct days on number of shoots/plantlets (Table 9). The highest number of shoots/plantlet (1.36, 2.00, 3.33 and 4.33 shoots/plantlets at 7, 14, 21 and 28 DAI) (Figure 10-13) at a concentration T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) was found from Granola, while the lowest number of shoots/plant (1.00 and 1.33 shoots/plantlets at 21 and 28 DAI) was found in Cardinal at a concentration T_6 (1.50 mg/l IBA +1.0 mg/l 2,4-D +0.5 mg/l BAP). More or less similar result was also found in variety Diamant at the same concentration (Table 9).



A B C

Figure 9. Maximum number of initiated shoot at 7 DAI on MS media supplemented with 0.25 mg/l IBA+0.125 mg/l 2, 4-D+0.5 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

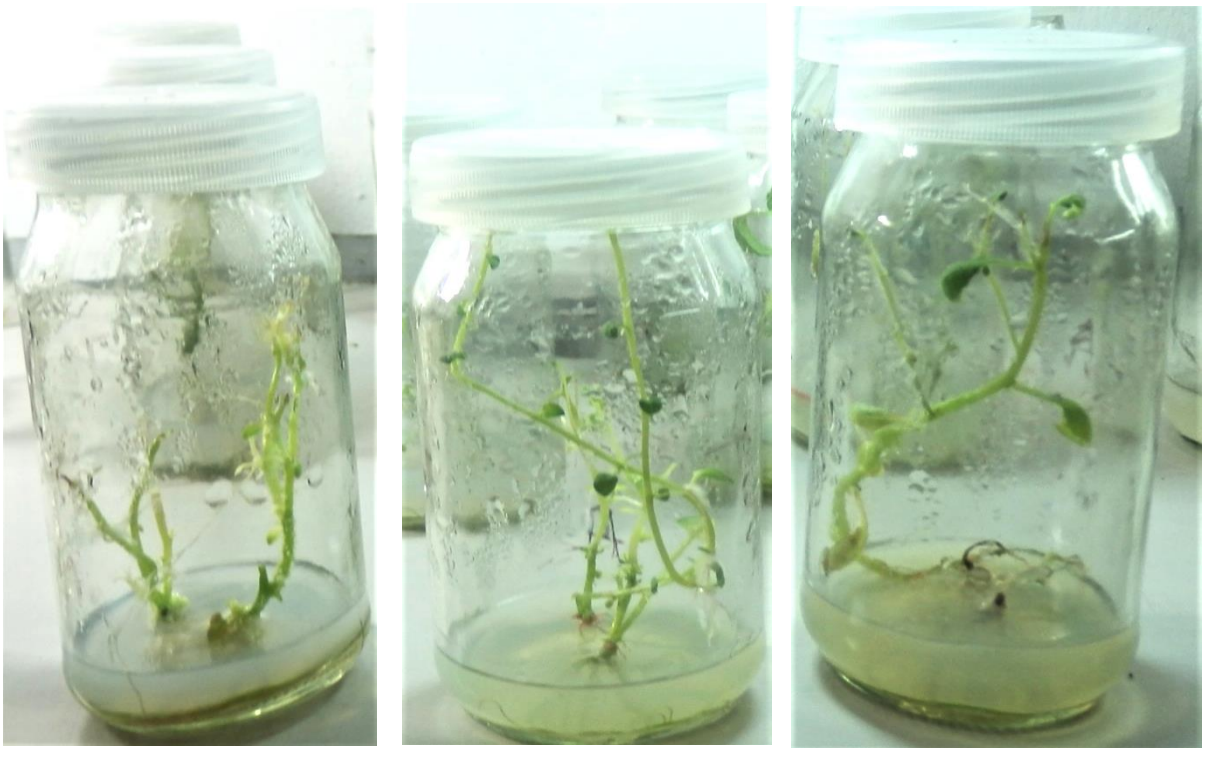


A B C

Figure 10. Maximum number of initiated shoot at 14 DAI on MS media supplemented with 0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

A B C

Figure 11. Maximum number of initiated shoot at 21 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)



A B C

Figure 12. Maximum number of initiated shoot at 28 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

4.2.6. Length of shoots/plantlet

The length of regenerated shoot per callus differ significantly among diverse potato genotypes. The effect of BAP was also observed by (Bhuiyan, 2013). Use of BAP in modified MS medium was reported well for plantlets development from meristem-tip culture in potato (Marani and Pisi, 2007). The length of shoots of the varieties was increased at 14, 21 and 28 DAI. The highest shoot length (0.52 cm) was recorded in Granola at 14 DAI and the shortest shoot length (0.19 cm) was recorded in Cardinal at 14 DAI (Table 9). The longest length of shoots/plantlet at 21 and 28 DAI (0.75 cm and 2.41 cm at 21 days and 28 days respectively) was found in variety Granola, whereas the shortest (0.19 cm) length of shoot was recorded at 14 DAI (0.36 cm and 1.07 cm at 21 days and 28 days respectively) in variety Cardinal (Table 10).

Table 10: Effect of different potato verities on regeneration of Length of shoots/plantlet and number of leaves/plantlets

Potato Varieties	Shoot length (cm)			Number of Leaves/plantlets
	14DAI	21DAI	28DAI	
Granola	0.52	0.75	2.41	10.2
Diamant	0.22	0.25	0.27	7.93
Cardinal	0.19	0.36	1.07	6.38
LSD	0.11	0.14	0.54	7.09
Level of significance	**	**	**	**

The Combined effect of unlike treatment at different concentrations of hormones displayed most significant variation at different days on length of shoots/plantlets (Table 11). The maximum length of shoots/plantlet (2.4 cm,5.86 cm, 6.90 cm and 9.32 cm shoots/plantlets at 7, 14, 21 and 28 DAI) (Figure 14-17) at a concentration T_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5

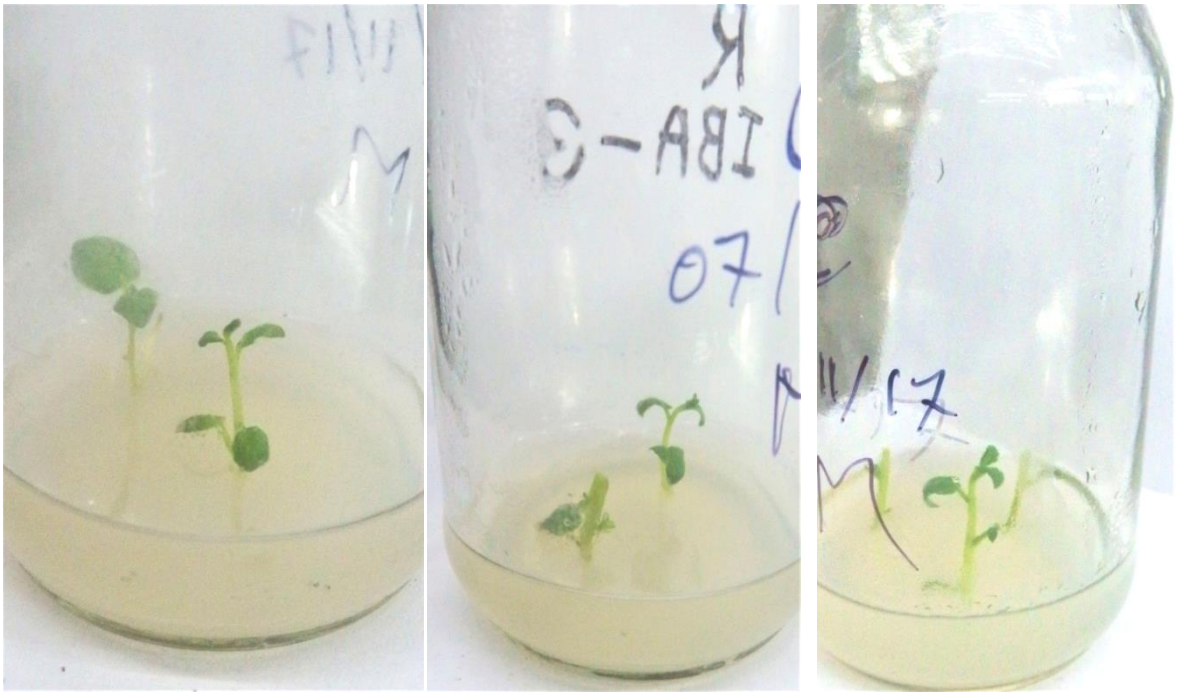
mg/l BAP) was originated from Granola, whereas the minimum length of shoots/plantlet (1.64 cm, 2.41cm, 2.77 cm and 3.10 cm shoots/plantlets at 7 days, 14 days 21days and 28 days respectively) was found in Cardinal at a concentration T_4 (0.25 mg/l IBA +0.37 mg/l 2,4-D +1.5 mg/l BAP). With the combination of T_5 (0.25 mg/l IBA +0.50 mg/l 2,4-D +2.0 mg/l BAP) no shoot formation occurred in variety Diamant (Table 11).

Table 11: Combined effect of different varieties and different hormones on length of shoots/plantlets and number of leaves/plantlets :

	Treatment	Length of shoots/plantlets (cm)				Number of Leaves/plantlets
		7 DAI	14 DAI	21 DAI	28 DAI	
Granola	T_1=Normal MS	2.14 b	2.265b	3.74 c	8.17 c	8.67 b
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	2.4 a	5.86 a	6.90 a	9.32 a	15.00 a
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	1.90 c	2.11 c	2.37 f	6.63 e	6.33 c
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	1.6133 d	2.11 b	2.61 e	4.00 f	5.33 c
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	0.51 e	1.63 d	1.88 g	3.31 g	6.00 c
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	0.00 f	1.31 e	2.96 d	8.01 d	8.67 b
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	0.00 f	0.75 f	4.11 b	9.03 b	8.33 b
Diamant	T_1=Normal MS	2.86 a	3.81 a	4.54 a	6.66 c	14.33 a
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	2.11 b	2.20 bc	3.11 c	7.53 a	11.33 b
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	1.74 c	2.14 c	2.21 e	5.46 e	4.00 d
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	1.71 d	2.54 b	2.92 d	3.28 f	6.67 c
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	0.00 e	0.00 d	0.00 g	0.00 g	0.00 e
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	0.00 e	0.00 d	1.73 f	5.66 d	6.67 c
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	0.00 e	0.00 d	3.20 b	7.31 b	7.33 c

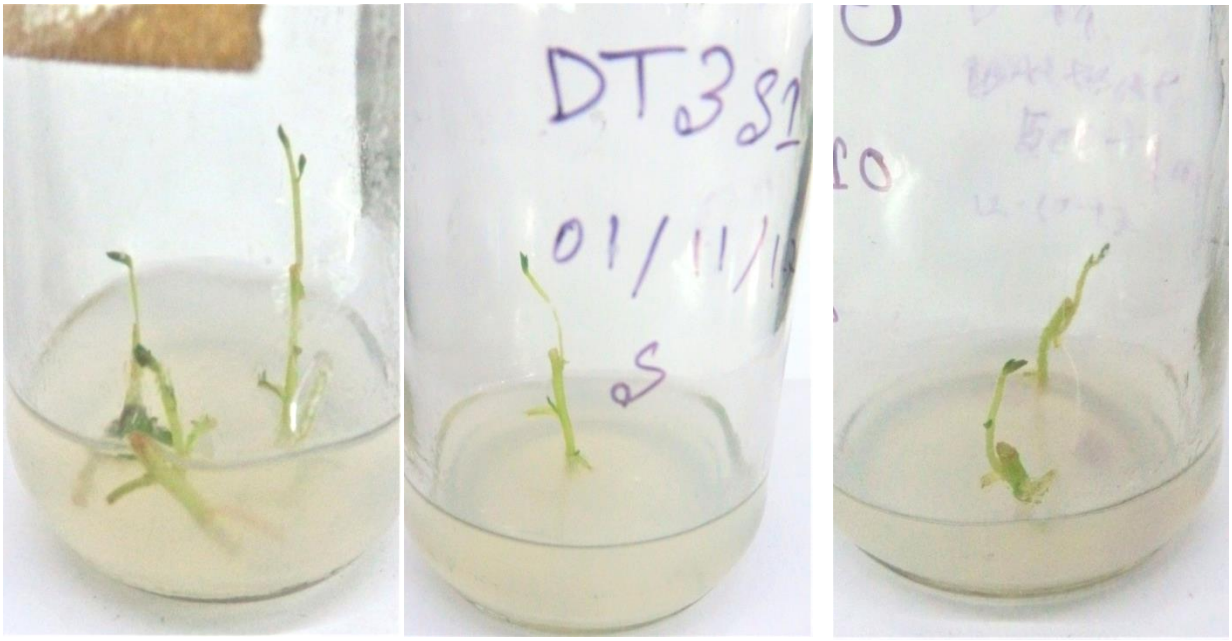
Table Continued

Cardinal	T_1=Normal MS	2.65 a	3.56 a	4.40 a	6.23 d	4.67 d
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	1.64 c	1.76 d	2.01 e	6.82 c	13.33 a
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	1.91 b	2.27 c	2.37 c	6.22 d	5.67 cd
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	1.64 c	2.41 b	2.77 b	3.10 f	5.67 cd
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	0.41 d	1.23 e	1.75 f	3.30 e	5.33 cd
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	0.00 e	0.00 f	1.36 g	6.91 b	6.33 c
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	0.00 e	0.00 f	2.12 d	7.62 a	7.67 b
	CV	0.74	0.43	0.43	0.14	0.11
	LSD	0.0153	0.0121	0.0179	0.0138	0.122



A B C

Figure 13. Maximum length of initiated shoot at 7 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

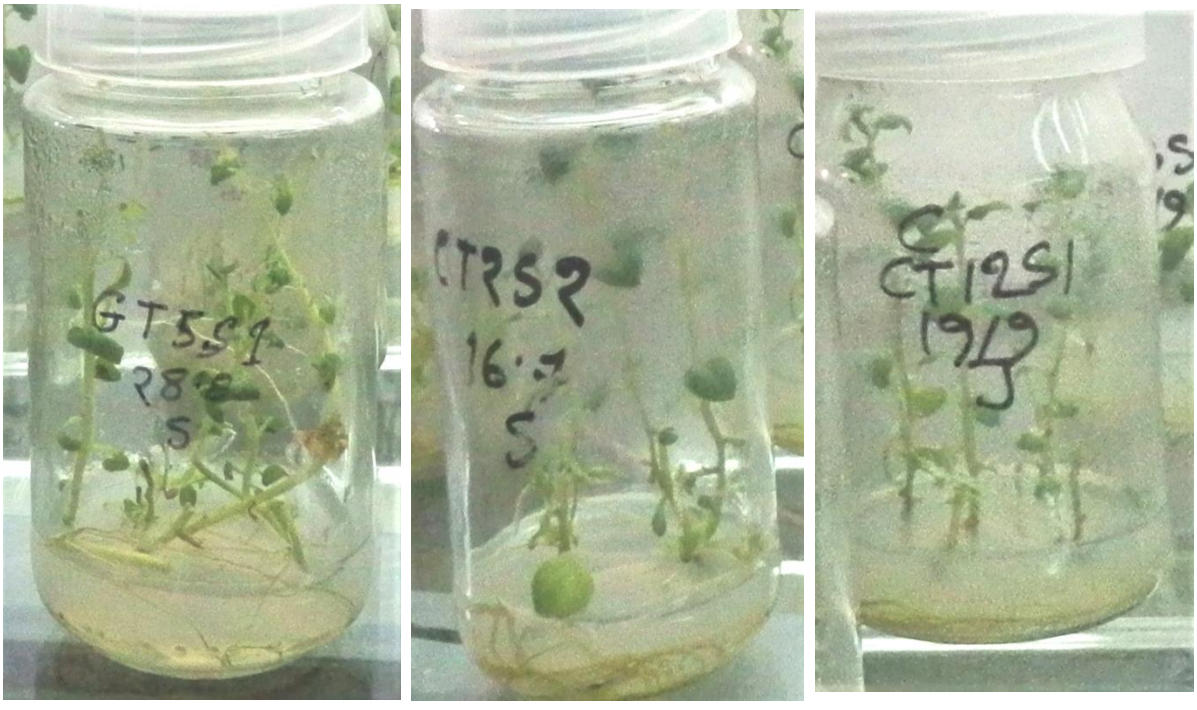


A B C

Figure 14. Maximum length of initiated shoot at 14 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

A B C

Figure 15. Maximum length of initiated shoot at 21 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)



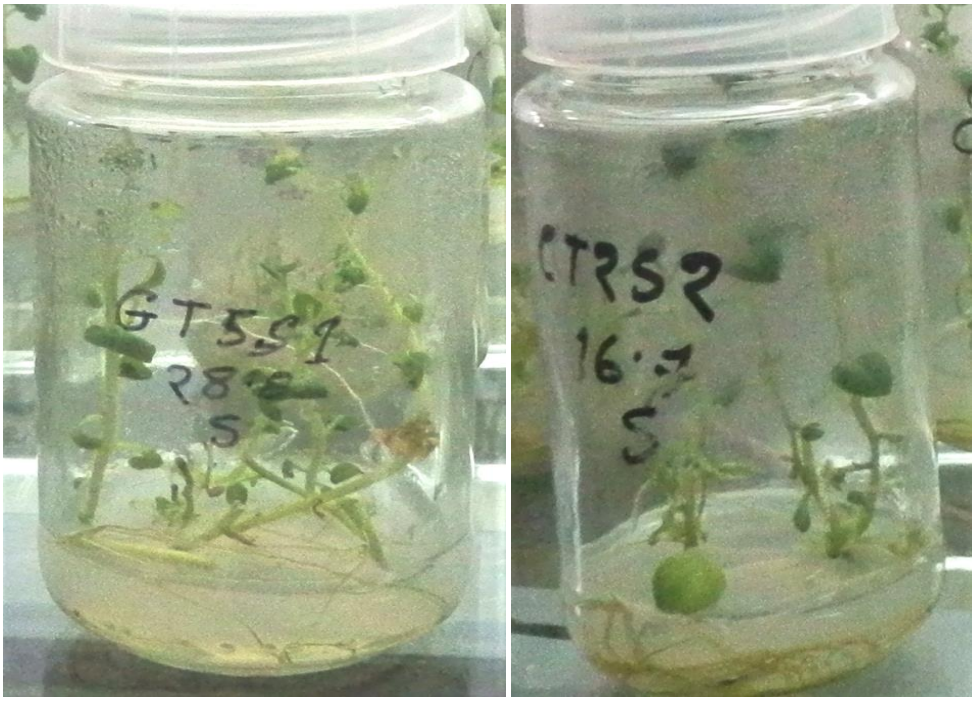
A B C

Figure 16. Maximum length of initiated shoot at 28 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

4.2.7. Number of leaves/ plantlets

Different potato varieties and concentrations of GA3 extensively influenced the number of leaves/ plantlet. Data was recorded at 21 DAI. The response of potato genotypes on number of leaves/ plantlets was noticed significant. Results presented in (Table 9) showing that the highest number of leaves/ plantlets was (10.2) filed in Granola and smallest number of leaves/plantlets was (6.38) recorded in Cardinal. During the experiment Diamant yielded (7.93) leaves/plantlets (Table 10).

The effect of three unlike treatments were executed Significant variations on number of leaves/plantlets have been presented in (Table 11) and (Figure 18). The greatest number of leaves/plantlets (15.00) was recorded in Granola at a combination T_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP) and the lowest number of leaves/ plantlets (4.00) was recorded in variety Diamant at T_3 (0.25 mg/l IBA+0.25 mg/l 2, 4-D +1.0 mg/l BAP) concentration. No leaves were found in Diamant variety at T_5 (0.25 mg/l IBA +0.50 mg/l 2,4-D +2.0 mg/l BAP) (Table 11).



A B

C

Figure 17. Maximum number of initiated leaves/plantlets at 28 DAI on MS media supplemented with 0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP Granola (A), Diamant (B) and Cardinal (C)

4.2.8. Days to root initiation

Days to root commencement was recorded after 7, 14, 21 and 28 days of cultured on MS media containing diverse concentrations of IBA, 2,4-Dand BAP. Results have been presented in (Table 12-13). All the three varieties were differed in days to root initiation.

The outcome of foremost effect of varieties on days to root induction have been presented in (Table 12). The days to root initiation differ significantly amongst the three varieties. Maximum period of days was recorded in variety Cardinal (21.67 days) where as the minimum days was noticed in variety Granola only (11.45 days).

Table 12: Effect of different varieties on days to root initiation and of length of roots/plantlet at different days after inoculation

Variety	Days to root initiation	Length of roots/plantlets			
		7 DAI	14 DAI	21 DAI	28 DAI
Granola	11.45	0.94	1.42	2.13	8.44
Diamant	15.76	1.11	1.82	3.52	6.35
Cardinal	21.67	0.44	0.61	1.44	5.98
CV	17	20	18	15	14
LSD	0.407	0.128	0.168	0.317	0.465

There was significant influence of different hormone combination and concentrations of IBA 2, 4-D and BAP on the days to root initiation (Table 13). The minimum (4.44 days) was observed in case of Granola on MS media with the combination of T_5 (0.25 mg/l IBA +0.50 mg/l 2,4-D +2.0 mg/l BAP). The maximum (28.88 days) was required by the variety Cardinal for root initiation on MS media without hormone (Fresh media).

Table 13: Combined effect of different varieties and different hormones on days to root initiation and length of roots/plantlet at different days after inoculation

	Treatment	Days of root initiation	Length of roots/plantlets			
			7 DAI	14 DAI	21 DAI	28 DAI
Granola	T_1=Normal MS	27.43	0.00 b	0.00 b	0.00 f	2.11F
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	20.01	0.00 b	0.00 b	1.98 c	5.64 b
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	20.00	0.00 b	0.00 b	1.88 d	6.55 a
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	21.56	0.00 b	0.00 b	1.31 e	2.11 f
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	4.44	1.24 a	2.54 a	3.10 a	3.98 c
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	20.34	0.00 b	0.0000 b	2.73 b	3.64 d
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	26.21	0.00 b	0.00 b	0.00 f	2.86 e
Diamant	T_1=Normal MS	26.63	0.00 b	0.00 b	0.00 d	1.99 g
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	26.67	0.00 b	0.00 b	0.00 d	3.47 c
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	21.34	0.00 b	0.00 b	1.34 c	3.31 e
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	21.33	0.00 b	0.00 b	1.49 b	2.10 f
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	4.67	1.55 a	3.06 a	3.55 a	4.00 a
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	25.69	0.00 b	0.00 b	0.00 d	3.34 d
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	24.64	0.00 b	0.00 b	0.00 d	3.71 b
Cardinal	T_1=Normal MS	28.88	0.00 b	0.00 b	0.00 d	1.87 f
	T_2=0.25 IBA+0.125 2,4-D+0.5 BAP	26.39	0.00 b	0.00 b	0.00 d	4.53 a
	T_3=0.25 IBA+0.25 2,4-D +1.0 BAP	20.67	0.00 b	0.00 b	2.19 c	4.32 b
	T_4=0.25 IBA +0.37 2,4-D +1.5 BAP	19.67	0.00 b	0.00 b	2.32 b	3.11 c
	T_5=0.25 IBA +0.50 2,4-D +2.0 BAP	5.33	0.99 a	1.85 a	2.43 a	3.11 c
	T_6=1.50 IBA +1.0 2,4-D +0.5 BAP	26.32	0.00 b	0.00 b	0.00 d	2.79 d
	T_7=1.50 IBA +2.0 2,4-D +1.0 BAP	28.33	0.00 b	0.00 b	0.00 d	2.25 e
	CV	2.60	1.71	0.50	0.72	0.39
	LSD	1.011	6.619	3.821	0.0115	0.0216

4.2.9. Length of roots/plantlets

Length of root was also displayed significant variations· amongst different potato varieties as data presented in (Table 12). The root length was recorded at 7,14, 21 and 28 DAI, the upper most length of root (0.94 cm, 1.42 cm, 2.13 cm and 8.44 cm respectively) in variety Granola. Whereas the minimum length of root (0.44cm, 0.61 cm, 1.44 cm and 5.98 cm at 7 days, 14 days, 21 days and 28 days respectively) was recorded in Cardinal. Medium root formation was observed in Diamant.

The effect of three diverse treatments was observed viewing significant variations on root length have been presented in (Table 13) and (Figure 19). Length of roots were counted at 7, 14, 21 1and 28 DAI at different concentrations. Maximum length of root (0 cm, 0 cm, 1.88 cm and 6.55 cm) was recorded in Granola. No root was formed at 7 and 14 days whereas at 14 days and 28 days roots were formed at a combination T_3(0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) and minimum length of root (1.87 cm) was recorded at 28 days in Cardinal at fresh media.

A B

C

Figure 18. Maximum length of roots with longest root initiated at 28 DAI on MS media supplemented with 0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP in Granola (A), Diamant (B) and Cardinal (C)

From on top of the results it is obvious that amongst all three potato varieties Granola gave highest values in respect of length of shoot (9.32 cm), number of shoots/plantlets (4.33) and root length (7.14 cm) and number of leaves/ plantlet (15.00) whereas Cardinal gave minimum values in respect of number of length of shoots/ plantlet (3.10 cm), number of shoots/plantlets(1.33) and number of leaves/ plantlet (4.00) was found in Diamant variety. Variety Granola showed maximum length of root (8.44 cm) and minimum length of root (1.87 cm) was found in Cardinal variety among all genotypes.

4.2.10. Experiment 3: Detection of Virus through Serological test (DAS-ELISA)

After shoot and root multiplication, for a serological identification/sero-diagnosis was done of the developed plantlets for virus detection. The Double Antibody Sandwitch Enzyme Llinked Immunosorbent Assay (DAS-ELISA) was done. The plantlets, all varieties except GT2S1 and GT6S3 were virus free, showed no development of color. The results of ELISA test are presented in (Table 14).

Table 14: DAS-ELISA test of potato plantlets for viruses detection

Sl. No.	Sample ID	Potato Viruses	Result
1.	GT1S1	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected
2.	GT2S1	PLRV, Potato Virus A, X, V	PVY, PVM, and PVS are detected
3.	GT2S3	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected
4.	GT5S1	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected
5.	GT6S3	PLRV, Potato Virus A, Y, X,M, S,	PVV detected
6.	GT7S1	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected
7.	CT2S2	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected
8.	CT12S1	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected
9.	DT3S1	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected
10.	DT4S1	PLRV, Potato Virus A, Y, X,M, S, V	Virus not detected

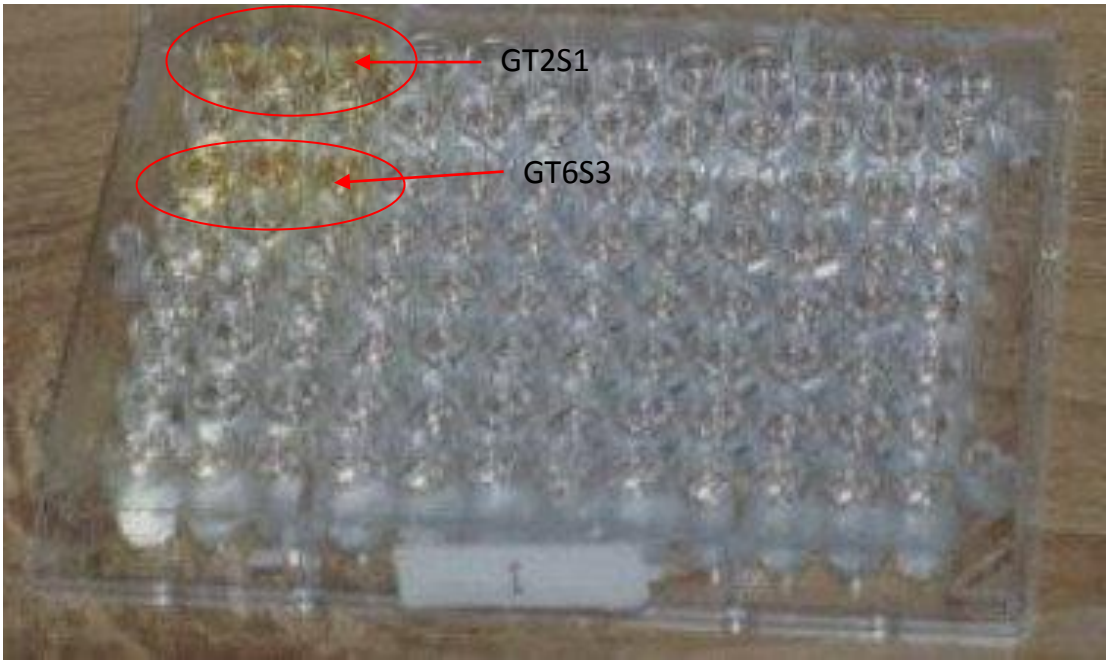


Figure 19. In ELISA test, development of yellow colour in two micro titer wells indicating presenceof virus and in other micro titers no development of colour indicating are freeness of the sample plantlets derived from meristem culture

4.3. *In vivo* hardening/acclimatization and establishment of plantlets in soil

The virus free plantlets with well furnished shoot root and leaves (preferably 28-32 days old) that attains height about 6-8 cm were driven out from the culture vials devoid of damaging any roots. The culture media attached to the roots was gently washed away from the roots with running tap water to prevent further pathogenic infections. Pot preparation was done for transferring of the virus free plantlets. For potting mixture of garden soil, sand and well decomposed cow dung were mixed up in a ratio (1:2:1). Then the mixture was autoclaved for sterilization purpose at 120℃ for 20 minutes at 1.16 kg/cm^2. After that small culture pots were filled with the soil for raising plantlets *in vivo* condition. Plantlets were then transferred to small pots containing soil. After transplantation immediately the pots containing plantlets were wrapped with moist polythene bags to restrain desiccation. The plantlets along with pots were kept under restricted environment in a growth chamber for 5-7 days to reduce

any sudden stroke. The inner part of the polythene bag was sprayed with distilled water to sustain high humidity of the plantlets at every 24 hours. Then 2-3 days later the polythene bag was removed and pots were transferred to net house and appropriate care was taken for highest growth and development of plantlets. Rooted plantlets were gradually acclimatized and successfully established in the pots under net house conditions (Figure 16). Survival rate of the studied three varieties were estimated. Maximum percentage of survival rate 81.48% observed in cv. Granola and minimum percentage of survival rate 73.07 found in cv. Cardinal (Table-15) (Figure 21).

Table 15: Survival rate of in vitro regeneration plantlets of three potato varieties

Acclimatization	Variety	No. of transplanted plants	No. of plants survives	Survival rate (%)
Initially small plastic pots used as growth chamber	Granola	27	22	81.48
	Diamant	27	20	74.07
	Cardinal	26	19	73.07
Subsequently when moved to net house	Granola	22	20	90.90
	Diamant	20	17	85.00
	Cardinal	19	17	89.47

Under net house maximum plantlets displayed vigorous growth and some less vigorous growth was also noticed. After another 15-18 days plantlet were transferred to field condition hardening in normal environment for mini tuber production. After initiation of new leaves and new buds plantlets were transferred to medium sized pots for proper hardening (Figure 22-23) with normal environment. At this stage virus free plantlets were watered with normal tap water and gradually plantlets were adapted to soil.

Figure 20. *In-vivo* acclimatization of regenerated plantlets and transplantation of healthy plantlets to the pots under net house conditions

A B

Figure 21. Maximum survival of regenerated plantlets and of healthy plantlets to the pots under environmental conditions A. Granola B. Cardinal

Figure 22. Hardening of regenerated plantlets from the pot under environmental condition

4.3.1. Harvesting of Potato mini tubers

Potato mini-tubers were harvested from pots of established meristem tissue culture healthy potato plants. Among the three varieties maximum amount of potato mini tubers per plant were collected from Granola variety as compared to Diamant and Cardinal. The harvested potato mini tubers have been showed in (Figure 24).

Yield performance of harvested virus free potato mini tubers were recorded. Several yield parameters viz. total no. of minitubers/plants, wt of minitubers, maximum noumber of minitubers and average wt of minitubers were used. Maximum weight (20 gm) of mini tubers obtained from cv. Granola and minimum weight (18 gm) found in cv. Cardinal. Highest number (12) of mini tuber yield from cv. Granola whereas Diamant gave the lowest (9) number of minitubers (Table 16).

Table 16: Yield performance of different potato varieties:

Sl. No.	Variety	Yield Parameters			
		Total no. of minitubers/plants	**Wt. of minitubers (gm)**	**Max. no. of minitubers**	**Average wt. of minitubers (gm)**
1	Granola	**15**	**20**	**12**	**20.67**
2	Diamant	**10**	**22**	**9**	**21.84**
3	Cardinal	**13**	**18**	**10**	**18.33**

A B

C

Figure 23. Harvested potato mini tubers of Granola, Diamant and Cardinal respectively

4.4. DISCUSSION

Potato (*Solanum tuberosum* L) ranks first among the vegetables in terms of production area and consumption. It produces more calories and protein per unit land with minimum time and water than most of the major food crops (Upadhya, 1995). In the last few decay, dozen of high yielding potato varieties were imported to Bangladesh and tried experimentally underneath restricted conditions previous to being recommended for general cultivation (Islam *et al.*, 2003). High yielding foreign potato varieties significantly enhanced the yield of potato crop in our country at the same time new fungal, viral, bacterial and nematode diseases are also imported which causes reduction in the yield and quality of tubers. Among the different biotic agents, plant viruses have the more chances to transmit through the vegetative planting materials like potato tuber and among the viral diseases *PLRV, PVY* and *PVX* are most problematic which have been estimated 10-90% yield losses here in Bangladesh. The virus-free replica created more dynamic haulm and about 10% higher yields, imposed to more tubers more freely than large ones (Karim *et. al.*, 2011*).*

4.4.1 Effect of GA3 concentration at 400 ppm/L on sprouting ability of three selected potato cultivars

In this study (experiment-1) effect of GA3 concentration at 400 ppm/l was observed on sprouting ability of three selected potato cultivars. The shortest (3 days) time was taken by Granola to induce sprouting, maximum (6) number of sprouts/potato was recorded in Granola. The highest (5) Number of sprouts/eye and longest (4.2 cm) length of sprout was induced by Cardinal and all the parameters were easily responded at 400 ppm GA3 treatments. Pang Wen Jing (2004) published a research article stating an experiment conducted on seed tubers of potato cv. Favorita which were subjected more or less similar GA3 treatments to investigate their effects which supported the present study.

4.4.2. Effect of 2,4-D, BAP and IBA in callus induction, shoot and root regeneration of selected three potato varieties from meristem tips

The effect of 2,4-D, BAP and IBA, in callus induction, shoot and root regeneration from meristem tip culture, of selected three potato cultivars were examined in experiment 2. In this experiment the best combination in respect of days to callus induction was studied. The maximum size of callus (0.74 cm) was obtained in the Granola variety at T_3 combination (0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP), within the minimum period of times (only 2.0 days). The results are commenced with one previous findings that was reported by Khalafalla *et al*. (2010) and Shahab-ud-din *et al.* (2011). They have carried out experiments to explore the effects of different concentrations of plant growth regulators and their combinations on callus induction of potato (*Solanum tuberosum* L.).

The variety Granola was also took minimum days (5.34 days) when cultured at T_3 combination (0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP) and produced the maximum number of shoots/plantlets (4.33). The maximum length of shoot (9.32 was also obtained in Granola cultivar at T_2 combination (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP). Zaman *et al.* (2001) found the effect of IBA at different levels of concentration are effective for manufacturing virus free potato plantlets with maximum plantlets height, highest number of shoots and leaves.

In case of root formation the minimum time (44.4 days) was required at T_5 combination (0.25 mg/l IBA+0.50 mg/l 2,4-D +2.0 mg/l BAP) that was also in Granola variety, which also obtained the longest (6.55 cm) root when cultured with the supplement at T_3 combination (0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP). In one previous study on the meristem culture of potato that was conducted by Ghaffoor *et al.,* in 2003 and they found the positive effect of different combination of IBA, NAA and IAA for maximum root length, and in the same experiment the incidence of *PLRV, PVX and PVY* were also analyzed through ELISA test for the production of virus free potato plantlets.

4.4.3. DAS-ELISA test for potato viruses detection

The results of DAS-ELISA test showed that all potato plantlets samples that derived from meristem culture were free from PLRV, Potato Virus, Y, X, A, M, S, V. Where few samples were showed the positive results against the potato viruses viz. PVY, PVM, PVV and PVS were detected. The results of DAS-ELISA test confirmed the absence of major potato viruses like PLRV, PVX, PVA. Holmes (1948), Limusset (1949) and Kassanis (195) also found that the 'Imperfect allocation of virus in plant body and the nearer to the tip of plant the minor the content of virus'.

The study of several kinds of potato varieties and on different potato viruses has exposed that the development of the meristem varies by kinds of plantlets and that the separation of meristem is somewhat tricky. The improvement of the separation and disinfection method was achieved by addition of culture medium and growth motivating substance the efficiency in culture improved by a long way, achieving approving results in plantlets. The elimination ratio of virus is higher because the size is smaller (0.3-0.5 mm). So it is always necessary to isolate into maristem as explant from the source held with a limit (0.3-0.5 mm in diameter) so that virus is eliminated completely.

CHAPTER 5

SUMMARY AND CONCLUSION

The experiment entitled " Callus Induction and Virus Free Potato Mini-Tuber Production through Meristem Culture" was carried out by taking the facilities of the laboratory of Biotechnology Department, SAU and the laboratories of Plant Tissue Culture, Molecular Biology and Plant Virology, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka-1207. Three dissimilar sets of experiment were conducted and protocols were flourished. The first experiment was carried out to explore sprouting efficiencies of three selected viz. Granola, Diamant and Cardinal potato varieties. The second experiment was carried out to study the callus initiation ability and successive plant regeneration using meristems of sprout tips as explants. The third experiment was conducted to detect the potato viruses through serological test (DAS- ELISA test) in potato plantlets grown from meristem culture. Study *in vitro* plant enlargement and establishment of meristem culture resultant single shoots and *in vivo* establishment of these genotypes.

The first experiment was begun with principal materials that was collected from TCRC, BARI (Bangladesh Agricultural Research Institute), Gazipur. Three popular potato Varieties of comparatively alike size, shapes and weight were choose and cleaned for meeting the experiment. For conducting this experiment 400 ppm/l GA3 concentration was used, that given the efficient effect on sprouting within a short time as reported in the previous research..

For conducting the second experiment 2,4-D, BAP and IBA was used as a shoot and root regenerating plant growth hormone. The concentrations of 2.4-D were used 0.25 and 1.50 mg/l, BAP were 0.125, 0.25, 0.37, 0.50, 1.0 and 2.0 mg/l and IBA were 0.25mg/l and 1.50 mg/l. Combined effect of these hormones were also studied. For combined effect study, seven levels of treatments (T_1, T_2, T_3, T_4, T_5, T_6 and T_7) were used to study the effects on three potato Varieties concerning

callus initiation, callus sized. To determine the effect of several treatments of the studied experiment the parameters were used and data were recorded on callus color, callus texture, days to callus induction and size of callus, days to shoot initiation, length of shoots/plantlets, number of shoots/plantlets, number of leaves/plantlets, days to root initiation/plantlets and length of roots.

Their survival percentage also investigated after insertion of meristem in MS containing different hormonal treatments. Maximum survival percentage was observed in all varieties.

The colors of callus were found whitish green, yellowish green, greenish in the all varieties. The callus texture of Cardinal was non-friable and other two cultivars were developed friable callus texture.

MS media supplemented along with T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP) induced callus in minimum days (2.0 days). T_7 (1.50 mg/l IBA+2.0 mg/l 2,4-D+1.0 mg/l BAP) combination produced callus and took greatest number of days (9.0 days) to commence callus. Diamant required maximum days (9.0 days) to initiate callus and Granola formed callus in very little period of time within (2.0 days) only.

Maximum size of callus was recorded (0.61 cm) in Granola whereas minimum callus size was recorded (0.27 cm) in variety Diamant. Best treatment for producing large size callus (0.74 cm) when meristem of Granola was inoculated in T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP) combination whereas minimum size of callus was recorded (0.18 cm) when meristem of Diamant was inoculated in T_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP) combination.

Hormonal treatments were used to initiate single shoot from callus and only 13.15 days which was minimum days required for single shoot initiation. Highest number of single shoot (3.02) having the longest shoot (2.41 cm) and

also the maximum number of leaf (10.2) was produced by Granola within 28 DAI. Variety Diamant produced shortest number of shoot (1.97) and lower number of leaf (6.38) was recorded in variety Cardinal in 28 DAI. Variety Granola produced root within minimum days (11.45 days) having the largest roots (8.44 cm).

Combined effect of different treatment and various levels of hormones showed potato genotypes on days required for shoot initiation revealed that Granola took minimum days (5.34 days)) for shoot initiation when cultured in T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP) combination and produced the highest number of shoots/plantlets (4.33 shoots/plantlets at 28 DAI), having the longest shoot (9.32 cm shoots/plantlets) at T_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP) concentration. The maximum number of leaves/plantlets (15.00) was recorded in Granola at a combination T_2 (0.25 mg/l IBA+0.125 mg/l 2,4-D+0.5 mg/l BAP) concentration. The minimum (4.44 days) was observed in case of Granola on MS media with the combination of T_5 (0.25 mg/l IBA+0.50 mg/l 2,4-D+2.0 mg/l BAP) concentration with the longest roots (6.55 cm) at 28 DAI when cultured with the supplement T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP) concentration.

The overall investigated results showed that variety Granola was the most effective and rapid responsive genotype compared to the other the genotypes to GA_3 treatment for sprouting, and sprouting days was decreased with the increasing concentration of GA3 hormone. The findings of the studied experiment also implied that potato genotypes Granola, Diamant and Cardinal can also micro propagated using plant hormones 0.25 mg/l IBA+0.25 mg/l 2,4-D +1.0 mg/l BAP for rapid callus induction, large sized callus.

In the present study MS media supplemented with T_3 (0.25 mg/l IBA+0.25 mg/l 2,4-D+1.0 mg/l BAP) for multiple shoot regeneration and T_5 (0.25 mg/l IBA +0.50 mg/l 2,4-D+2.0 mg/l BAP) was showed to be most successful combination for speedy root development. It was noticed that auxin (IBA and 2,

4-D) are most responsive in combination with cytokinin (BAP) and produced callus in plenty.

Successive culture of sole shoot was done in MS media containing 1.0 gm/l BAP to obtain vigorous virus free plantlets for quick shoot multiplication. Among all potato Varieties Granola showed on the whole better performance in plant establishment from meristem tip culture to establishment of plants in environmental conditions. Plantlets were taken to BCSIR, for ELISA test which confirmed that two samples of Granola (GT2S1 and GT5S1) were infected with viruses (PVY, PVS, PVM and PVV) after meristem culture but rest of the samples gave virus negative results.

For acclimatization and hardening virus free plantlets were shifted from culture media to small pots containing disinfected soil with small holes. Survival rate differed (Table 14) from 81.48% to 73.07%. Maximum survival rate was recorded in Granola while it was minimum in Diamant. After transplanting of virus free plantlets grown in pots to field condition of soil survival rate was highest 90.90% filed in variety Granola and minimum 85.00% recorded in Diamant variety. Virus free potato varieties were survived in extreme field condition.

In the present study yield performance of the harvested virus free potato mini tubers were also recorded. Maximum weight (20 gm) of mini tubers obtained from cv. Granola and minimum with the highest number (12) of mini tuber yield from cv. Granola.

The protocol flourished from the experiment could successfully be used in large scale for virus or other disease free and healthy planting materials of potato production. Therefore, it can be concluded that Virus free mini tubers of potato can be grown in by meristem tip culture and successfully achieved higher yield with better grade of potato. The findings of the protocol can also be used in genetic and breeding program for improvement of disease free potato.

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APPENDICES

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

Components	Concentrations (mg/L)
Macronutrients *I* 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 three 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	2	92.825	287.2	2.74	31.925
Factor B	1	105.458	76.167	9.401	3.125
AB	1	23.858**	12.34	0.838**	0.725**
Error	8	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 three 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	2	56.133**	0.112**	1.479**	0.594**
Factor B	6	126.933**	0.059**	0.579**	0.239**
AB	12	18.867**	0.044**	0.165**	0.059**
Error	17	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	2	2.78**	176.102**	1.067**	429.947**	62.773NS
Factor B	6	1.015**	50.268**	5.544**	87.075**	60.73NS
AB	12	0.346**	22.364**	1.380**	61.405**	15.829NS
Error	17	0.055	5.976	0.261	8.128	2.336

** = Significant at 1 % level

* = Significant at 5% level

NS= Non Significant

Appendix-V: Published abstract from my research

Abstract of the Annual Plant Tissue Cul. & Biotech. Conf.

BSRI, Ishurdi, Pabna, 6-7 April, 2018

13 Virus Free Potato Mini-Tuber Production through Meristem Culture

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Experiments were conducted with a view to establish a protocol for virus free mini-tubers production, *in vitro,* using potato meristem as explant collected from potato sprouts of three popular potato varieties Diamont, Cardinal, Granola. In the present study four levels of GA3 (100, 200, 300 and 400 ppm) was used to assess the influence on sprouting abilities in three selected potato verities. The maximum sprouting efficiency was observed in 400ppm GA3 treatment within short period of time in Granola variety. The effect of different combination and concentration of IBA and BAP was used along with fresh MS media to inoculate meristems of potato sprouts. IBA concentration were 1mg/L, 1.5mg/Land BAP concentration were 0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2mg/L. The effect of combined hormones was also studied. The Diamond variety showed the maximum callus size (1.98cm) within a very short period of time (7 days) while treated with IBA at the concentration of 1.0 mg/L and BAP at 1.5 mg/L. Granola meristem inoculated in hormonal treatment showed the best results regarding minimum days required to shoot initiation (6 days) followed by Cardinal (8 days) and Diamond (9 days). The highest number of shoots/plantlet (4 shoots/plantlet) and the longest plantlet (6.3cm) was found in Granola variety followed by Cardinal (3.0 shoots/plantlet and 5.8cm) and Diamond (3 shoots/plantlet and 4.5cm). Maximum numbers of leaves were also found in Granola variety (16 leaves) in the treatment combination of 1.0 mg/L IBA with 1.5 mg/L BAP. Granola gave maximum performance in respect of maximum number of root (15 roots/plantlets) within a short time of root initiation (20 days), followed by Cardinal (7.0 roots/plantlets) of root initiation (21 days) and Diamond (6 roots/plantlets) of root initiation (23 days). Granola produced the longest (6.59 cm) roots/plantlet. Rooted plantlets were gradually acclimatized and successfully established in the field. However, in overall observations in this study, Granola showed better performance from meristem tissue culture and all plants were found normal and free from potato viruses that were tested through ELISA test.