CALLUS INDUCTION AND *IN VITRO* PLANT REGENERATION OF BARI RELEASED POPULAR POTATO (*Solanum tuberosum* L.) CULTIVARS

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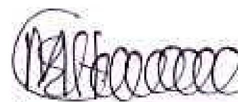

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This is to certify that thesis entitled, ***"CALLUS INDUCTION AND IN VITRO PLANT REGENERATION OF BARI RELEASED POPULAR POTATO (Solanum tuberosum L.) CULTIVARS"*** *submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE*** *in* ***GENETICS AND PLANT BREEDING****, embodies the result of a piece of bona fide research work carried out by Miss* ***NUSHRAT JAHAN MOON****, Registration No: 05-01837 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

Dated: December, 2010
Place : Dhaka, Bangladesh

....
(Prof. Dr. Naheed Zeba)
Supervisor

DEDICATED TO
MY
BELOVED PARENTS

SOME COMMONLY USED ABBREVIATIONS AND SYMBOLS

The following abbreviations have been used in this thesis

Abbreviations	Full word
°C	Degree Celsius
@	At the rate of
%	Percentage
1N	1 Normal
2,4-D	2,4-Dichlorophenoxyacetic acid
a.i	Active ingredient
Agric	Agriculture
BAP	Benzyl Amino Purine
BADC	Bangladesh Agricultural Development Corporation
BBS	Bangladesh Bureau of Statistics
BARI	Bangladesh Agricultural Research Institute
BAU	Bangladesh Agricultural University
cm	Centimeter
Cont'd	Continued
CRD	Completely Randomized Design
cv.	Cultivar
CIP	International Potato Centre
Conc.	Concentration
DAI	Days after inoculation
DMRT	Duncan Multiple Range Test
DW	Distilled Water
et al.	And others
etc.	Etcetera
FAO	Food and Agricultural Organization
Fig.	Figure
g	Gram
g/L	Gram per liter
GA_3	Gibberelic Acetic Acid
ha	Hectare
ha^{-1}	Per hectare
h.	Hours
$HgCl_2$	Mercuric Chloride
i.e	id test(That is)
IAA	Indole-3-Acetic Acid
Intl.	International
J.	Journal
mg	Milligram(s)
mg/L	Milligram per liter
ml	Milliliter
MS	Murashige and Skoog

LIST OF ABBREVIATIONS AND SYMBOLS (Cont'd)

Abbreviations	Full word
μM	Micro mole
NAA	Napthalene Acetic acid
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
No.	Number
NS	Non Significant
ppm	Parts per million
pH	Negative logarithm of hydrogen ion concentration (-log[H+])
PGRs	Plant Growth Regulators
SAU	Sher-e-Bangla Agricultural University
Sci	Science
TCRC	Tuber Crop Research Center
Univ.	University
Viz.	Namely
w/v	Weight/volume
ZR	Zeatin Ribosome

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The author sincerely and modestly wish that this thesis work could help respected researchers who are involved in tissue culture all over the world and can contribute to the scientific knowledge of plant regeneration, which are widely used in tissue culture.

December, 2010.
Place: SAU, Dhaka

The Author

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CALLUS INDUCTION AND *IN VITRO* PLANT REGENERATION OF BARI RELEASED POPULAR POTATO (*Solanum tuberosum* L.) CULTIVARS

ABSTRACT

By

NUSHRAT JAHAN MOON

The present study was undertaken in the Genetics and Plant Breeding Laboratory and the Tissue culture laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, during the period of January 2011 to December 2011, to investigate the *in vitro* performance of three Bangladesh Agricultural Research Institute (BARI) released popular potato cultivars viz. Granola, Diamant and Asterix using potato sprout as explant. Five levels of GA_3 (50, 100, 200, 300 and 400 ppm/L) were used to find out the best concentration for sprout initiation, and it was revealed that GA_3 at the rate of 400 ppm/L was the best concentration for maximum sprouting within short period of time. The effect of different combination and concentration of NAA and BAP on *in vitro* callus induction and plant regeneration was also studied. The concentrations for NAA were 1.0 mg/L and 1.5 mg/L, and for BAP were 0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L. The combined effect of both the hormones were also studied. The variety Diamant produced callus in the shortest period of time (4.46 days). It took minimum days (3.53 days) for callus induction in the treatment of 1.0 mg/L NAA+1.5 mg/L BAP. The largest size (2.10 cm) and the highest weight (2.54 gm) of callus were noticed in the variety Diamant with the treatment 1.5 mg/L NAA within 28 DAI. In case of shoot initiation, variety Granola took minimum time (13.17 days) followed by Diamant (13.63 days) and Asterix (15.09 days). The variety Granola produced the maximum number of shoots/plantlet (3.00 shoots/plantlet) and the longest (6.76 cm) shoots/plantlet followed by Diamant (2.12 shoots/plantlet and 6.51 cm) and Asterix (1.91 shoots/plantlet and 5.68 cm), respectively. Variety Granola also produced the maximum number of leaves/plantlet (20.0 leaves) with the treatment 1.5 mg/L NAA+1.5 mg/L BAP within 28 DAI. The variety Asterix took the minimum (12.73 days) days to root initiation followed by Granola (15.01 days) and Diamant (15.48 days). In treatment 1.5 mg/L NAA+0.5 mg/L BAP required the minimum (0.93 days) days to root initiation and also the number of roots/plantlet (14.53 roots/plantlet at 28 DAI) was the maximum. When variety and treatment took under consideration, the treatment 1.5 mg/L NAA+0.5 mg/L BAP with variety Diamant produced the maximum (19.40 roots/plantlet) and Granola produced the longest (14.42 cm) roots/plantlet respectively. The regenerated plantlets were transferred successfully in plastic tray and subsequently to soil. The variety Granola survived at high rate in growth chamber and in open atmosphere. Among all the varieties, Granola showed the best performance from callus induction, plant regeneration to *in vivo* establishment.

CHAPTER 1
INTRODUCTION

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CHAPTER 1
INTRODUCTION

Potato (*Solanum tuberosum L.*) is one of the most important vegetable crops in Bangladesh as well as world contexts. It has multipurpose use in daily consumption and also industrial purpose and is one of the prominent crops capable of nourishing the world's population. It is one of the most economically important annual vegetable crops in Solanaceae family and originated in the central Andeans area of South America. In respect to land under cultivation potato is the third major subsistence crop in Bangladesh (Sarker *et al.*, 2002). In Bangladesh total area under potato crop has been estimated at 4,60,197 hectares and 18.093 M. Tons/ha of potato were produced during 2010-2011 (BBS, 2011). The average yield of potato in Bangladesh is 19.69 t/ha which is very low compared to many potato growing countries like Netherlands (44.7 t/ha), the USA (44.6 t/ha) and Germany (42.3 t/ha) (FAO, 2008). However potato production has to be increased, even with the current rate of demand. Among the reasons of lower yield of potato, non-availability and higher price of quality seed are important. In Bangladesh, Bangladesh Agricultural Development Corporation (BADC) supplies about 7-8 thousand tones of high quality seed potato which is only 3.8 to 4.3% of total seed requirement of the country. Some private companies are also supplying few thousand tones of commercial grade seed tubers. The rest amount of tuber planted every year for the production of potato in the country is the tubers produced as table potatoes (Siddique, 1999). To make Bangladesh sufficient in food, extensive research work has been performed for the improvement of economically important crops that exist in the country.

Potato and many other vegetatively propagated crops are frequently characterized by their inability to produce seed due to presence of one or more factors: incompatibility, dichogamy, abnormal seed and seedling development, seed dormancy and environmental factors. These affect flowering and seed setting. Presence of these factors possesses margins on the use of breeding techniques for improvement. Potato can be propagated sexually (by botanical seed, also called true potato seed) and asexually (vegetatively) by means of tubers (Otroshy, 2006). In spite of having problems in conventional breeding, most of the potato varieties available now a days have been developed through natural selection and conventional breeding which is very lengthy process. There are two major problems associated with conventional

clonal multiplication of potato-seed stocks. Firstly, low multiplication rate in the field that takes as long as 7 to 12 years, causing a lack of flexibility to the changing needs of the end-users. Secondly, high susceptibility of potato to viral bacterial and fungal diseases (Dobranszki *et al.*, 2008). Routine production of disease free seed tubers is necessary to maintain adequate yields. That is why reliable and pathogen free propagation has been started all over the world (Hossain, 1988). Among these, the important role is played by the *in vitro* vegetative micro propagation, since this method is pathogen free. Diseases free good quality seed and pathogen free planting materials are possible to produce through this technique (Hossain, 1994) and to conserve the germplasm of rare and endangered species (Fay, 1992). Nowadays, micro- propagation is the alternative to conventional propagation of potatoes. Micropropagation, used as standard methodology for production of disease free (virus free) seed potatoes. This system involves two stages: 1) *in vitro* multiplication and production of *in vitro* plantlets, 2) production of minitubers in the greenhouse (Otroshy, 2006).

In Bangladesh, beside low supply of quality seeds, the plants are infected by different viruses, bacteria, fungi, viroids and many insect pests. Among these leaf roll, leaf curl, late blight, early blight etc are the main problems for potato production. Potatoes are found to be infected with more than 20 viral diseases, of them PVX and PVY depress tuber yield up to 80% and may destroy whole crop in combination with others. As a result yield, quality and storability of potato reduce drastically every year. As potatoes are vegetatively propagated crop, the seed borne pathogens of previous year pose a serious threat to the potato production of the following year. Researchers showed that some viruses can decrease the yield by 40% singly and in combination with other viruses, the loss is 90% (Siddiqui *et al.*, 1996). Plant tissue culture offers an efficient method for production and rapid propagation of pathogen-free material and germplasm preservation of plants to overcome this unwanted situation. *In vitro* plant regeneration has become a popular and useful technique and being applied to solve the problems of many agricultural crops. Creation of novel germplasm through techniques of tissue culture and gene transfer holds great potential for improving the quality, resistance to diseases and agronomic characters of potato (Jayasree *et al.*, 2001). A reproducible protocol for *in vitro* regeneration is a prerequisite for varietal improvement through genetic engineering. Type of explants, media compositions, growth conditions, and genotypes affect callus induction and plant regeneration. So,

the best growth condition, suitable explants and genotypes are needed to identify for large-scale utilization in biotechnology. Hence, it is necessary to identify which variety is more prone to *in vitro* culture. It is important to standardize the protocol of explants response for callus induction and plant regeneration through callus of potato cultivars.

Both callus induction and plant regenerations from explants require the presence of appropriate combinations and concentration of plant growth regulators in the cultured media (Ehsanpour and Jones, 2000; Fiegert *et al*., 2000; Ahn *et al*., 2001). Growth regulators play an important role in potato regeneration. Individual hormone has its on effect on regeneration. Combine effect of rooting and shooting growth regulator is needed to study for better regeneration of potato. Therefore, the present investigation was carried out to identify the best hormonal combination and concentration for potato regeneration. As well as to detect the best response to regeneration among the different varieties of potato.

The callus formation was first reported in 1951 (Steward *et al*., 1951) leading to plant regeneration in 1975 (Lam, 1975). It is important due to produced genetic variability, which is very important in breeding programme. In addition, it is easy to produce huge amount of *in vitro* plantlet via callus. But the response of explants for callus induction and regeneration need to be standardized. Therefore, the aim of the present study is to establish an effective protocol to investigate the *in vitro* callus induction ability of potato with optimum concentration of plant growth regulator that can produce clean and decontaminate *in vitro* microplants of potato from three BARI released potato cv. Granola, Diamant and Asterix to maintain stocks for *in vitro* multiplication and rapid plant regeneration in Bangladesh.

The present experiment aims at developing an efficient high frequency plant regeneration system with the following objectives:

1. To study the performance of *in vitro* callus induction of three BARI released popular potato cultivars.
2. To investigate the callus induction and plant regeneration ability of potato with optimum concentration of NAA and BAP.
3. To establish an effective regeneration protocol of BARI released potato with optimum concentration of plant growth regulator.
4. *In vitro* multiplication of plantlet in a short period of time.

CHAPTER 2
REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Potato is one of the prominent crops capable of nourishing the world's population. It is one of the most economically important annual vegetable crops in Solanaceae family (Keeps, 1979). Nowadays plant tissue culture techniques are being applied for rapid clonal propagation of potato. However, many research works on *in vitro* micro propagation of potato have been conducted in different countries of the world. There are various factors that effect *in vitro* callus proliferation and plant regeneration such as position of the explants on the plant as well as size of explants, genotypes of the explants, physiological state of the donor plants and explants, concentration of the nutrients and plant growth regulators in the culture medium and environment under which cultures are grown i.e. light, temperature and humidity (Gregory and John, 1998).

A lot of many studies have been made earlier too in view callus induction and regeneration of potato. In this chapter, the most pertinent literature relevant to the present study is reviewed here under separate headings.

2.1. Concept of potato tissue culture

Propagated by tubers that are used by farmer is a conventional system. Potato production with seed tuber is constrained by the accumulation of pathogen, physiological decline and low multiplication rates. Seed tuber is most expensive input in potato production. At least 35-40% total cost of potato production is covered by seed tuber and it is very lengthy process. Nowadays plant cell tissue culture techniques are being applied for rapid multiplication of plantlet production of potato. Tissue culture or cell culture is the process where cells are grown and maintained in a controlled environment such as a laboratory, outside their natural and original source. Cell culture is a vital technique in many branches of biological research. *In vitro* produced disease free potato clones combined with conventional multiplication methods has become an integral part of seed production in many countries (Naik *et al.*, 2000).

Callus is an amorphous mass of loosely arranged thin walled undifferentiated parenchyma cells arising from the proliferating cells of parent tissue (Dodds and

Roberts, 1990). In plant biology, callus cells are those cells that cover a plant wound. Murashige and Skoog (1962) reported that the nutritional requirement for optimal growth of *in vitro* tissues may very with varieties. To induce callus formation, plant tissues are surface sterilized and then plated onto *in vitro* tissue culture medium. Plant hormones, such as auxins, cytokinins, and gibberellins, are supplemented into the medium to initiate callus formation or somatic embryogenesis.

2.2. *In vitro* callus induction of potato

Yasmin *et al.* (2003) conducted an experiment to observe the effect of NAA and BAP on callus formation and regeneration from leaf and internodal segment explants. Five levels of each of NAA (0, 1.25, 2.5,5 and 10 mg/L) and BAP (0, 0.5,1, 2 mg/L) were applied to MS media for callus induction and plantlet regeneration. Twenty explants were cultured in each combination. Leaf showed better performance in callus induction and plantlet regeneration. This highest percentage of callus (95%) and minimum time (8.13 days) were recorded with 2.5 mg/L NAA+2 mg/L BAP. The percentage of regeneration (80%) was with 2.5 mg/L NAA+2 mg/ L BAP among the combinations. It was also observed that callus was derived from leaf plantlets in a shortest period of time (23.68 days) compared to that from stem (28.96 days).

Haque *et al.* (2009) conducted an experiment with different concentrations of 2, 4-D and kinetin showed highly significant differences for length and weight of callus formed except interaction of callus weight. Leaf explant appeared to be best of all for callus length and weight when 1.0 mg/L 2, 4-D + 0.25 mg/L kinetin concentration was used. Similarly, different explants versus different concentrations of BAP/GA_3/IAA showed significant differences for shoot length and leaf number/plantlet and also for root length. However, interaction term confirmed node and node/internode explants produced better results in shoot length and number of leaves/plantlet when concentrations 1 .0 mg/L BAP + 0.1 mg/L GA_3 and 1.0 mg/L BAP + 0.2 mg/L GA_3, 1.0 mg/L BAP + 0.4 mg/L GA_3, respectively, were used. Similarly, internode explants produced better results for root length after 21 days plantlet when concentration of 1.0 mg/L IAA + 0.25 mg/L GA_3 was used. Shoot tip explants also produced better results in root length after 28 days $plantlet^{-1}$ when concentrations 1.0 mg/L IAA + 0.25 mg/L GA_3 were used.

A study has been made by Anjum and Ali (2004) to observe the effect of culture medium on shoot initiation. Attempts were made to regenerate shoots from calluses of different origin (tuber, leaf, internodal) on different media in two cultivars, Cv. Maris Piper and Cv. Desiree of potato. Media containing zeatin was found to be more effective than the one containing BAP for shoot regeneration. Also Cv. Maris Piper showed better response than Cv. Desiree.

Elaleem *et al.* (2009) conducted an experiment to investigate the effects of different concentrations and combinations of growth regulators on callus induction and plant regeneration of potato (*Solanum tuberosum* L.) cultivar Diamant. The tuber segments were used as explants and cultured on MS medium supplemented with different concentrations of NAA, 2,4-D, BA and Thidiazeron (TDZ) alone and 2,4-D in combinations with BA for callus induction. The best degree for callus formation (6.0) was obtained on MS medium supplemented with 2, 4-D alone at 3.0 mg/L or 2, 4-D in combination with BA both at 2.0 mg/L. MS medium containing 5.0 mg/L TDZ was the best for days to shoot initiation, the highest percentage of callus with shoot (81%) and the highest number of shoot/callus (3.4). Callus derived shoots were rooted most effectively in half-strength MS medium containing 0.5 mg/L IBA.

Khatun *et al.* (2003) carried out an experiment on nodal segments of Diamant cultivar of potato from *in vitro* grown plantlets for callus induction and regeneration on MS semisolid medium supplemented with different concentrations of 2,4-D, NAA, BAP alone and NAA with BAP. Highest 90% of callus formation was observed in MS+2.5 mg/L 2, 4-D. The second highest 83.33% callus induction was recorded in MS+5.0 mg/L BAP. Maximum percentages (70%) of calli induced shoots were observed in MS medium fortified with 5.0 mg/L BAP+0.1 mg/L IBA. The regenerated shoots were rooted on MS and ½ MS medium containing different concentration of IBA and maximum rooting response was achieved in ½ MS+1.0 mg/L IBA.

A study was conducted by Omidi and Shahpiri (2003) to determine the effects of growth regulators (2, 4-D and kinetin), cultivar, explant and light on callus induction in potato. They found significant effect of 2, 4-D combined kinetin and their interactions on the frequency of callus induction and roots on the callus. The effect of cultivar, explant and their interaction on frequency of callus induction was not

significant, while the effects of these factors on the initiation time of callus induction was significant. Callus was mostly induced in leaf explants under dark conditions, but was induced in internode explants under both dark and light conditions.

An effective procedure has been developed by Jayasree *et al*. (2001) for including somatic embryogenesis from leaf cultures of potato cv. Jyothi. Leaf sections were initially cultured on 2, 4-D+ Benzyladenine (BA) and NAA+BAP supplemented Murashige and Skoog (MS) media. Nodular embryogenic callus developed from the cut ends of explants on media containing 2, 4-D and BA, whereas compact callus developed on media containing NAA and BA.

More *et al*. (2001) evaluated MH5 and Hiobras-6 (BB-6) to determine their effects on embryogenic callus formation of potato cv. Desiree. The best callus formation was observed in medium -1 supplemented with 0.1 mg MH-5+ 0.01 mg BB-6/L and in medium -2 supplemented with 0.001 mg MH5 and 0.01 mg BB-6/L. There was no callus formation when MH5 and BB-6 were used as substitutes for 2, 4-D. Calls formation was observed when MH5 and BB-6 were used as kinetin substitutes.

Shirin *et al*. (2007) was conducted an experiment with the internodal and leaf explants of four potato cultivars *viz*. Diamant, Multa, Atlas and Lalpakri. Where callusing response of both types of explants and for Atlus internodal esplants was the best in 3.0 mg/L 2, 4-D containing MS media among 2,4-D, NAA alone and with BAP combinations. MS medium containing 4.0 mg/L KIN+0.5 mg/L NAA was the best for maximum shoot regeneration from the internode and leaf derived calli in most of the cultivars. The regenerated shoots were rooted in MS_0 medium and successfully transplanted to the field.

Effect of growth regulators for callus induction of potato (*Solanum tuberosum* L.) genotypes (Christian and Roclas) was studied by Andreea *et al*. (2009). It was revealed that the best callus induction was for MS medium supplemented with 1mg/L 2, 4-D and 0,5 mg/l BAP for both the genotypes.

The aim of the study of Turhan (2004) was to establish an effective protocol for callus induction from the potato genotypes (*Solanum tuberosum* L.) used and to investigate whether the transferred oxalate oxidase enzyme or transformation procedure has any

effect on callus induction of transgenic lines of cultivar Desiree and Maris Bard. The results showed that the effects of genotype and medium on measured characters were highly significant. In order to compare overall performance of transgenic lines of each cultivar with their parental cultivars (non-transgenic), orthogonal comparisons were used and indicated the significant differences between transgenic and non-transgenic genotypes. The significant differences between the cultivars and their transgenic lines expressing oxalate oxidase enzyme indicated that the transformation procedure had a significant effect on their callus induction and growth.

Ali *et al.* (2007) was conducted an experiment to standardize the *in vitro* regeneration protocol of six potato varieties viz. Raja, Diamant, Cardinal, Heera, Granola and Lalpakri. The highest percentage (62.60%), the maximum height of plantlet (12.90 cm), the maximum number of leaves/plantlet (13.90) were produced from internodal segments derived calli of Heera with 6.0 mg/L BAP and the lowest percentage (29.80%), the minimum height (4.68cm) of plantlet, the minimum number of leaves/plantlet (7.83) was produced found in leaf segments derived calli of Lalpakri with 2.0 mg/L BAP at 90 DAI. The maximum (103.03) days to shoot initiation was noted in calli of leaf segments of Lalpkari with 2.0 mg/L BAP and the minimum (53.93) days was found in internodal segments derived calli of Heera with 6.0 mg/l BAP.

Callus was initiated from primary root explants and mature embryos of cv. Bala on modified Linsmaier & Skoog (LS) medium by Fiegert *et al.* (2000). Calli initiated on medium with sucrose as sole carbon source regenerated shoots at a frequency of 20-23% on LS medium containing 1 mg/L IAA +4 mg/L kinetin. Whereas root callus lost its ability to initiate shoots by 75 days and embryo callus by 100 days in culture, the rhizogenic potential of both callus types was retained beyond 100 days. Root and embryo calluses of Bala initiated on 2% sucrose+3% sorbitol or mannitol and then transferred to the same regenerating medium as before produced shoots over a period of 600 days. Shoots were produced in 15-20 days with 49-61% frequency.

Kollist *et al.* (1994) studied the callus formation ability of 21 potato varieties on MS medium with different concentrations of growth regulators and sugars and concluded that regenerative ability depends on variety and the composition of the medium.

where the most effective media was 0.1 mg/L IAA and 6 mg/L benzyladenine. Callus from 6 of the varieties regenerated shoots on suitable media, while others formed only single shoots and 2 varieties were unable to regenerate shoots.

Martel and Garcia (1992) conducted an experiment with potato cv. Sebago. Leaf discs were cultured on MS media with 30 mg sucrose/L, to which 0-0.1 mg NAA/L+0-4 mg BAP were added to the initial medium, and 0-4 mg BAP+ 1 or 5 mg GA_3 for the shoot proliferation medium. They noticed that NAA was essential for callus formation. The amount of callus formation increased with the increasing concentrations of both NAA and BAP with best result obtained with highest concentrations of both the growth regulators. They observed that both BAP and GA_3 were necessary for shoot formation. Shoot formation occurred more rapidly with higher BAP concentration.

Asma *et al.* (2001) studied the effect of different concentrations (1, 2, 3, 4 and 5 mg/L) of GA_3 and BAP on the *in vitro* multiplication of nodal fragments and stem segments of potato cv. Desiree. The maximum shoot length (8.96 cm) was obtained when 4 mg/L GA_3 was applied. The number of nodes was not significantly affected by any of the GA_3 concentrations used in that study. The maximum number of shoots (14) was obtained when 2 mg/L BAP was applied.

Fomenko *et al.* (2000) studied the dynamics of callus formation in potatoes. MS medium supplemented with α-NAA was selected fro tuber explants and a medium with 2, 4-D and kinetin for leaf and stem explants. Proteins and nuclic acids were analyzed during callus development.

Callus formation was investigated by Fomenko *et al.* (1998) in tuber, stem and leaf explants of potato hybrid 78563-76 and cultivars Rosinka and Otrada on MS medium supplemented with various growth regulators. Different explants and different genotypes reacted differently to the growth regulators in the medium in terms of callus formation. Protein and nucleic acid metabolism during callus formation also differed with genotype and explant type.

Callus formation of 5 potato genotypes with different genetic origin was analyzed by Dobranszki *et al.* (1999) on 5 different media to select the optimal treatment. Both induction and the rate of callus growth were strongly influenced by genotype and medium and significant 2nd order interactions were proved statistically. The best undifferentiated growth of friable calli on leaf explants was observed after 4 weeks on medium containing 0.25mg kinetin and 5.0 mg 2, 4-D/l.

Alphonse *et al.* (1998) Cultured leaflet explants, tuber discs and callus tissues from micropropagated plantlets of potatoes cv. Cara, Desiree, Diamant and Spunta on a variety of media supplemented with different growth regulators. Regeneration from leaflets was best on Nitsch and Nitsch medium, while Gamborg medium was best for tuber discs. Spunta showed the highest regeneration from leaflets, while than from tubers discs were greatest with Diamant followed by Desiree. Supplementation of Murashige and Skoog medium with 1 mg Benzyladenine, 1 mg IAA and 10 mg GA_3 was the best combination of growth regulators for regeneration from leaflets, while 0.4 mg IAA, 0.4 mg GA_3, 0.8 mg Kinetin and 1.0 g Casein hydrolysate/L was best for tuber discs. Medium containing 5 mg, 4-D and 2 mg kinetin/L was best for callus induction.

Uddin *et al.* (2011) investigated 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 Modified MS medium supplemented with different concentrations of 2, 4-D, NAA, BA, 2, 4-D in combinations with BA and NAA in combination with BA for callus induction. They revealed that, comparatively a massive amount of callus was formed on MS medium supplemented with 2, 4-D alone at 3.0 mg/L. The degree of callus was best at higher concentrations of NAA and BA. 2, 4-D in combination with BA at 2.0 mg/L produced considerable amount of callus. In case of NAA in combination with BA the degree of callus formation was best at concentration 1.0 mg/L. It was concluded that 2, 4-D is the best option for induction of callus among the other hormones used.

The variation in mutant lines of the three potato varieties Cardinal, Diamant and Desiree was studied by Ahmad *et al.* (2010). All the three varieties showed different callus induction response against different tested media with varying concentrations

of plant hormones i.e., Cardinal (37.8%), Diamant (32.8%) and Desiree (42.8%) respectively. Similarly the regeneration efficiency of the callus from microtuber, root, leaf node and internode explants of Cardinal, Dimant and Desiree with respect to different concentrations of growth regulators such as Colchicine and Sodium azide (0.1mM, 0.2mM, 0.3mM, 0.4mM and 0.5mM) was determined. Cardinal, Diamant and Desiree gave 35.33, 31.33 and 40.33 percent response respectively.

2.3. *In vitro* plant regeneration of potato

Hoque (2010) was conducted an experiment to develop a protocol for rapid plant regeneration in potato. The newly emerged sprout of six potato cultivars were used as explants. Varietal response on *in vitro* regeneration under different hormonal concentration and combination were studied. MS medium supplemented with 0.5, 1.0, 1.5, 2.0, 4.0 mg/L of KIN and IAA in combination of both were employed for *in vitro* regeneration. Among the different treatments the combination 2.0 mg /L of KIN and IAA showed the best response to multiple shoot and root regeneration and also took minimum time for regeneration. The interaction between hormonal concentration and varietal potentiality for all the parameters showed significant difference at 5% level of probability. The *in vitro* regeneration and multiplication potentiality was the highest in the variety Granola followed by Cardinal and Diamant.

Rahman *et al*. (2010) studied on the regeneration ability via callus induction using leaf disc of five tobacco varieties. Explants were cultured on MS medium supplement with different concentrations and combinations of plant growth regulators. Among the varieties used, the highest percentage of callus induced in 2.0 mg/L Kinetin and 2.0 mg/L IAA. Shoots were induced from calli cultured on the same medium. Maximum shoot formation from leaf discs was on medium supplemented with 2.0 mg/L Kinetin and 2.0 mg/L IAA. It was also revealed that, the rooting response of regenerated shoots was in using ½ MS medium with IBA (0, 0.5, and 1.0 mg/L). The highest root formation was found in MS medium supplemented with 0.5 mg/L IBA.

Jun *et al*. (2005) studied the effect of auxin, GA_3 and BAP on potato shoot growth and tuberization was investigated under *in vitro* condition. The shoot length of potato explants increased with the increasing of concentrations (0.5-10 mg.dm-3) of IAA treatment especially with the addition of GA_3 (0.5 mg.dm-3), but was inhibited by

BAP (5 mg.dm-3). The root number and root fresh weight of potato explants increased with the increasing of IAA levels either in the presence of GA_3 or not. However, no root was observed in the treatment IAA+BAP, instead there were brown swollen calluses formed around the basal cut surface of the explants. The addition of GA_3 remarkably increased the fresh weight and diameter of calluses.

Shi *et al.* (2004) carried out an experiment with 3 factors at 5 levels was adopted to screen optimal plant hormone combinations that could induce stem segments to differentiate shoots directly at 25 degrees C, 16 h photoperiod and 1500 lx light intensity. The 3 factors were GA, NAA and BAP. Stem segments of virus free seedlings *in vitro* of potato cv. Super White were used as the explants and the MS medium was used as basic culture medium. Effects of the 3 plant hormones on callus differentiation was ranked as NAA>BA>GA_3. The optimum combination was 0.25 mg NAA + 1.5 mg BA + 7 mg/L.

Molla *et al.* (2011) carried out an experiment to find out a suitable growth regulator and its optimum concentration for direct regeneration. Seven different concentrations of BAP, six different concentrations of Thidiazuron (TDZ) and eight different concentrations of Zeatin riboside (ZR) were tested separately for *in vitro* direct regeneration of potato along with GA_3 (0.2 mg/L) and IAA (0.01 mg/L). Among the different concentrations of BAP, TDZ and ZR, MS medium supplemented with 3 mg /L of BAP, 0.3 mg /L TDZ and 5 mg /L of ZR showed very good shoot initiaiton. Moreover, among the BAP, TDZ and ZR, ZR showed the very good performance in respect of direct regeneration. Within the different concentration of ZR, MS medium supplemented with 5 mg/L showed the best performance in respect of shoot initiaiton from both internode and leaf explants.

Ahloowalia (1982) studied a procedure for plant regeneration from callus culture of potato, *Solanum tuberosum* L. Calli were induced from 1–2 mm long shoot apices of potato cultivars Cara and A25/19 on half-strength MS medium (half-MS) supplemented with 3.2 IAA mg/L, 1.0 kinetin mg/L and 0.5 2, 4-D mg/L. 60% explants produced nodular calli on this medium within 30 days. Calli differentiated into shoot-primordia when subcultured on half-MS medium supplemented with 0.5 mg 2,4-D and 1.0 zeatin mg/L. Differentiated calli on half-MS medium without

growth hormones produced complete plantlets which were cloned on the same medium and transferred into soil.

An experiment was evaluated by Khalafalla *et al.* (2010) for plant regeneration from callus culture of potato. Calli were induced from 1.0 cm^2 tuber segment of potato cultivar Almera on MS medium supplemented with different levels (1.0-5.0 mg/L) of 2, 4-D. The highest degree of callus formation (3.0) and 100% of explants produced nodular calli on MS medium within 7-12 days when supplemented with 2.0-5.0 mg/L of 2, 4-D. Calli were differentiated into shoot primordia when subcultured on MS medium supplemented with 1.5 -5.0 mg/L of thidiazuron (TDZ) and 2.0-5.0 mg/L of BA. The best result for number of shoot/callus (3.3 ± 0.3) and longest shoot (0.8 ± 0.1) were obtained by using TDZ at 5.0 mg/L. Callus derived shoots were rooted most effectively in full-strength MS medium containing 1.0 mg/L IBA. The success of plant tissue culture for *in vitro* culture of potato was encouraged by acclimatization of the plantlets in the greenhouse conditions.

Esna-Ashari and Villiers (1998) reported that shoots and callus were formed from tuber discs of potato cv. Desiree when grown *in vitro* on Murashige and Skooge (MS) basal medium supplemented with 2,4-D and/ or BAP. Callus was formed in MS medium with 1 mg/L BAP+0.5 mg/L 2,4-D. Callus and roots were formed on MS with 1 mg/L BAP plus more than 0.5 mg/L 2,4-D and shoots were formed directly on tuber discs cultured on MS medium with 1 mg/L BAP without the addition of 2,4-D.

An experiment was conducted by Rodriguez *et al.* (2000) for one step regeneration system, using leaf explants of potato cultivars Diacol Capira (DC) and Parda Pastusa (PP). The effect of different ratios of auxins and cytokinins added to MS basal medium (supplemented with 30 g/L sucrose, 0.5 g/L thiamine, 1 mg/L GA_3, 40 mg/L ascorbic acid and 1.7 g/L phytagel and pH of 5.7) was investigated. All leaf explants from DC treated with zeatin riboside (3 mg/L) and in dole+3-acetic acid+ IAA (1 mg/L) and all leaf explants from PP treated with zeatin riboside (3 mg/L) induced regeneration, producing green and morphologically normal plants.

An experiment was conducted by Hansen *et al.* (1999) that shoot regeneration on explants from different leaves and leaflets to potatoes cvs. Pasmo, Folva and Oleva.

Explants were excised from glasshouse grown plants and grown for 6 days on callus induction medium with indole-3-acetic acid or 2, 4-dichlorophenoxyacetic acid. Explants were then transferred to auxin free shoot regeneration medium with gibberellic acid and 6-benzyladenine or zeatin. By using the optimum combinations and concentration of plants growth regulators by excision of explants from particular regions of proximal leaflets from newly unfolded leaves, shoot regeneration frequencies of 44.6% were obtained for cv. Pasmo and 32.1% from cv. Folva.

An experiment was conducted by Zel *et al.* (1999) where petioles, internodes and leaf explants in combination with different plant growth regulators, specially different concentration of zeatin riboside (ZR), were tested. Shoot regeneration was most successful on callus derived from internode tissue cultured on induction medium supplemented with 2.5 mg ZR, 0.2 mg NAA, and 0-0.02 mg gibberellic acid GA_3/L for 2 weeks and then transferred to a shoot initiaiton medium containing 2.5 mg ZR/L.

In vitro regeneration was assessed Hamdi *et al.* (1998) in potato cvs. Nagore, Desiree and Superior. Different explants such as leaves, tubers and microtubers and different regeneration media differing in their hormonal composition were tested to increase the efficiency of the process. Callus induction rates, number of shoots and number of regenerated plants were determined for each explants, culture medium and cultivar. Leaves were the best explants according to the observed regeneration rate, using an MS medium containing glucose (30 g/L), NAA(0.02 mg/L) Zeatin roboside (2 mg/L) and GA_3 (0.02mg/L). Tuber and microtuber explants reached higher regeneration rates in an MS medium with sucrose (30 g/L). IAA (0.5 mg/L) and zeatin riboside (3.5 mg/L).

An experiment was carried out by Ali *et al.* (2006) to observed callus induction and subsequent regeneration potentiality of potato varieties. Leaf segments of three potato varieties namely Diamant, Cardinal and Granolla were used as explants for callus culture and subsequent plant regeneration. Explant was cultured on MS medium containing different concentrations of NAA and BAP. The best callus (95.00) was observed in Diamant with media containing 1.00 mg/L NAA and 4.0 mg/L BAP and it required minimum number of days (8.75). The highest percent of regeneration (75.00) and shoot/callus (4.00) observed in Diamant in combined with media

containing 1.00 mg/L NAA and 4 mg/L BAP. Best rooting was also observed in same variety and same growth regulators combinations.

Effect of TDZ on several *In vitro* growth parameters in *Solanum tuberosum* cvs. Desiree and Cardinal were observed by Sajid and Aftab (2009). Shoot apices (1.0 cm each) from both the cultivars were separately inoculated on full strength MS basal media as well as on MS full strength supplemented with different concentrations of TDZ. MS full strength medium was found to be the best for *in vitro* micropropagation.

The present investigation was carried out aiming by Elaleem *et al.* (2009) to develop a technique for rapid *in vitro* micropropagation of plants. Nodal explants prepared from proliferating shoots of established axenic cultures of four potato cultivars viz Diamant, Alpha, Almera and Agria were used. Explants were incubated on MS medium supplemented with different concentrations of thiadizuron (TDZ) and BA alone or in combinations with NAA. Nodal explants responses to BA and TDZ were cultivar-dependent for number of shoot per explant. The highest number (5.4 shoots /explant) of shoots/explant was obtained for Almera explant cultured on MS medium supplemented with 3.0 mg/LTDZ in combination with 0.1 mg/L NAA. Regenerated shoots were rooted on MS medium with or without auxins. The longest root (8.8±1.3) was induced from shoot cultured on MS medium lacking growth regulator. The highest number (35.0±1.3) of roots/shoot was obtained on MS medium supplemented with IBA at 1.0 mg/L. Regenerated plants were successfully acclimatized and eventually transferred to the green house with 100% survival rate.

Endogenous levels of IAA were determined by Pal *et al.* (2011) in the internodal explants of Indian tetraploid potato cultivars (cvs) viz., Kufri Sutlej and Kufri Giriraj. The callusing response was inhibitory to shoot morphogenesis. Inclusion of an established anti-auxin, 2, 3, 5-tri-iodobenzoic acid (TIBA) in the regeneration medium facilitated a high frequency adventitious shoot regeneration response with lower cytokinin levels. MS medium containing TIBA at 2.5 mg/L and 0.25 mg/L zeatin evoked a 100% regeneration response. However, in cv K.Giriraj, which had lower levels of endogenous IAA, 80% regeneration response was recorded in an extended period on a medium containing 0.5 mg/L TIBA and 0.1 mg/L zeatin.

Although, TIBA and zeatin induced shoot bud formation, it failed to support sustained growth of the regenerated shoots in cv K.Giriraj. Hence, 0.01 mg/L NAA with a relatively higher concentration of zeatin (1.0 mg/L) were used for sustained shoot regeneration.

Micropropagation of four varieties of potato viz. Kufri Badshah, kufri Chandramukhi, Kufri Jyoti, Kufri Sindhuri was achieved by Jatinder *et al.* (2000) through the production of minitubers. Shoot nodal segments (1.0 cm) from *in vivo* grown plants were excised, surface sterilized with mercuric chloride (0.1%) and cultured on half strength MS medium. The shoots obtained *in vitro* were multiplied through subculturing after three weeks on fresh medium having same composition. Five times shoot multiplication per cycle was achieved in all the four varieties. The plantlets were rooted in liquid half strength MS medium with IBA (1.0 mg/1). Complete plants thus obtained were hardened and transferred to soil. These green house grown plants produced minitubers after 2 1/2 months.

2.4. Effect of growth regulators on root initiation and regeneration

Sanavy and Moeini (2003) was conducted an experiment with different concentration of NAA and BAP to observe the best concentrations of auxin and cytokinine for root formation. The modified solid (MS) medium without NAA and BAP was found to be best for the formation of roots and shoots. Addition of BAP and NAA in the medium decreased shooting and rooting, respectively of single node cultures.

An experiment was conducted by Sanavy and Moeini (2003) with solid MS medium supplemented with 0.25 mg/L GA_3, 0.01 mg/L NAA that showed significant differences between different pH levels in respect of its ability to root and shoot initiaiton in plantlets produced from the single nodes of two cultivar potato (*Solanum tuberosum* L.) varieties after subjecting them with thermotherapy. Overall pH 5.5 was the best for all the traits. Low and high levels of pH from 5.5 were found to reduce the growth and rooting of single nodes. The reduction was more definite at low levels than high levels of pH.

Maximum shoot regeneration in two indigenous potato (*Solanum tuberosum* L.) was observed by Sarker and Mustafa (2002) on MS semi-solid medium supplemented with

1.0 mg/L BAP and 0.1 mg/L GA_3. Among the two varieties, namely Lal Pakri and Jam Alu the former showed best response in terms of number of shoots/explant, nodes/shoot and shoot length. Half strength of MS containing 0.1 mg/l IAA was found to be best for root induction from the excised shoots.

In vitro shoots of *Solanum tuberosum* cv. Spunta, were sub cultured on liquid MS medium containing different combination of BA or Kinetin by Shibli *et al.* (2002). Significant reduction in stem and internode length was observed by increasing BA and Kinetin concentrations. BA resulted in increased number of proliferated shoots and number of roots. Single node cuttings were rooted on solid media containing NAA, IBA or IAA with or without GA_3. *In vitro* rooted shoots were successfully acclimatized to *in vivo* condition. Cuttings of 3 cm from glass house plantlets were successfully rooted by treating with 1.0mg/L IBA+0.5 mg/L IAA for five seconds.

Sidikou *et al.* (2003) in their study observed the production and management of microtubers using 10 varieties of potatoes. The selected genotypes were micropropagated *in vitro* in media containing different concentrations of sucrose at 2-12% and BAP at 0-5 mg/L. Shoots were formed after one week. All cultivars showed 100% regeneration. The frequency of regeneration raised with increasing concentrations of sucrose (8%, 10%, 12%) and BAP (5 mg/L).

From the above review of literature it appears that both callus induction and *in vitro* plant regeneration of potato are controlled mainly by three factors such as explant including genotype, culture media and culture condition.

CHAPTER 3
MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

The effect of variable concentrations of NAA and BAP on *in vitro* callus induction and plant regeneration using potato sprout and their multiplication was conducted in the Genetics and Plant Breeding Laboratory and the Tissue Culture Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, during the period January 2011 to December 2011. Three experiments were conducted to fulfill the objectives of the present study.

Experiment 1: Sprouting potentiality of three BARI released potato under different concentrations of GA_3 treatment

Experiment 2: Callus induction and plant regeneration of three potato varieties supplemented with different concentrations of NAA and BAP

Experiment 3: Acclimatization and establishment of plantlets in soil

These experiments were done in different steps:

1. Sprout initiation of the potato tuber by application of different doses of GA_3
2. *In vitro* callus induction from sprout of three BARI released potato varieties with the supplementation of different concentration of NAA and BAP.
3. *In vitro* plant regeneration of three potato varieties with the supplementation of different hormone concentration.
4. *In vitro* multiplication of the plantlets
5. Transfer of the *in vitro* plant in the net house
6. *In vivo* acclimatization of the *in vitro* plantlet
7. *In vivo* transfer of the potato plantlet to soil.

3.1. Experimental materials

3.1.1. Plant materials

Experimental materials are sprouts of three BARI released popular potato cultivars (Plate 1) viz.

1. Granola (BARI Alu- 13)
2. Diamant (BARI Alu- 07)
3. Asterix (BARI Alu-25).

3.1.2. Sources of plant materials

All of these potato tubers were collected from Tuber Crop Research Center (TCRC), Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. Disease free potato tubers were sprouted in the dark condition at room temperature with different concentrations of GA_3.

Plate 1: Primary materials. A. Granola, B. Diamant and C. Asterix

3.2. Plan of work

3.2.1. GA_3 treatment

- Collect different varieties of potato from TCRC, BARI, Joydebpur, Gazipur
- Wash them thoroughly with running tap water for several times to remove soil from the potato
- Then wash them with liquid detergent for cleaning purpose
- Wash several times with running tap water to remove detergent
- Now wash with distilled water
- Soak the potato tuber into liquid GA_3 at the concentration of 400 ppm/L for 15 minutes
- Pour the GA_3 in spray container
- Regular spray of the tuber for get immediate result twice in a day
- To find out the best concentration of GA_3 different potato varieties were selected for applying five concentrations of GA_3 treatments (50,100, 200, 300 and 400 ppm/L).
- After 4-5 days sprout was initiated from the tuber
- Of this GA_3 treatment, 400 ppm/L concentration found the best for sprout initiation, because it required less time for sprout initiation.

3.3. Culture media

Success of any experiment depends on the culture media, hormone combination, tissue and employing cell. Murashige and Skoog (1962) medium were used with different hormonal supplements as culture medium for callus induction and root regeneration. The composition of MS medium has been presented in Appendix 1. Hormones were added to MS media as per treatment of the experiment. For the preparation of media, stock solutions were prepared at the beginning and stored in the refrigerator at 4 ± 1°C. The respective media were prepared from stock solutions.

3.4. Preparation of the stock solutions

The first step in the preparation of the medium is the preparation of stock solutions of the various constituents of the MS medium. As different media constituents were required in different concentrations, separate stock solutions for the macronutrients, micronutrients, Fe-EDTA (Iron stock), vitamins and growth regulators were prepared separately for ready use.

3.4.1. Stock solution of macronutrients (stock 1)

Stock solution of macronutrients was prepared with 10 times the final strength of the medium in one liter of distilled water (DW). Ten times the weight of the salts required for one liter of medium weighted accurately. Dissolve all the macronutrient one by one except $CaCl_2$. The stock solution of $CaCl_2$ should be prepared separately in order to avoid precipitation. And in this way, dissolved all the salts thoroughly in 750 ml of distilled water and final volume was made up to one liter by further addition of DW. The stock solution was poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use.

3.4.2. Stock solution of micronutrients (stock 2)

A stock solution of all the micronutrients with 100x concentration is generally prepared. Since copper and cobalt are required in very small quantities, it is preferable to first make a separate stock solution of those two salts (100*) and then an appropriate volume can be pipetted and put into the main micronutrient stock solution. This stock solution was also stored in refrigerator at 4°C.

3.4.3. Stock solution of iron (Fe-EDTA) (stock 3)

Iron-EDTA should be added fresh and it was made 100 times the final strength of the medium in one liter DW. Here, two constituents, $FeSO_4.7H_2O$ and Na_2EDTA, were dissolved in 750 ml of DW in a conical flask by heating in a water bath until the salts dissolved completely and final volume was made up to one liter by further addition of DW. This stock should be stored in an amber color bottle or a bottle covered with an aluminum foil and stored in refrigerator at 4°C.

3.4.4. Stock solution of vitamins and growth regulators (stock 4)

The following vitamins were used in the present study for the preparation of MS medium. Myo-inositol (Inositol),

Nicotinic acid (Vitamin B_3),

Pyridoxin HCl (Vitamin B_6),

Thiamine HCl (Vitamin B_1),

Glycin.

Each of the vitamins except myo-inositol were taken at 100 times of their final strength in measuring cylinder and dissolved in 400 ml of distilled water. The final

volume was made up to 1000 ml by further addition of distilled water. This stock solution was also labeled and stored in a refrigerator at 4°C.

3.4.5. Stock solution of hormones and growth regulators

All the hormones are not soluble in water. Solubility of hormones are given in Table 1. To prepare the stock solution of the hormones, 10 mg of the hormones was placed on a clean beaker and then dissolved in a few ml of particular solvent and then water can be slowly added to make the requisite volume. The mixture was then collected in a 100 ml measuring cylinder and was made up to 100 ml with DW. Concentrations of compounds can be taken as mg/L or molarity. The solution was then poured in to a conical flask and stored at 4°C in a refrigerator.

Table 1: Molecular weight and solubility of some hormones

Growth regulators with group	Abbreviation	Molecular weight	Solubility
Auxin	NAA (α-Napthalene acetic acid)	186.2	1N NaOH Or 96% Ethanol
Cytokinin	BAP (Benzylaminopurine)	225.3	1N NaOH

3.4.6. Preparation of other stock solutions

3.4.7. Preparation of IN NaOH:

I. Put Weigh 40 g NaOH pellets
II. Put this pellets in a dry 1 L volumetric flask
III. Add slowly 900 mL distilled water and stir until dissolved
IV. The flask in a thermostat at 20 ^{0}C and maintain for 1 hour
V. Add distilled water up to the 1 L-mark and mix the closed bottle.

3.4.8. Preparation of 70% Ethanol

I. In a 100 ml measuring cylinder 70 ml 99.9% ethanol was poured.

II. Double distilled water was poured up to the level of 100 ml.

III. Store the solution in a sterilized glass bottle

IV. This solution was made fresh each time before use.

3.5. MS Media preparation

To prepare one liter of MS medium, the following steps were followed:

1. 500 ml double distilled water was taken into 1 liter beaker
2. 100 ml of stock solution of macro-nutrients, 10 ml of stock solution of micro nutrient, 10 ml of stock solution of Fe-EDTA and 10 ml of stock solution of vitamins and growth regulators were added in this 500 ml double distilled water
3. 30g of sucrose was dissolved in this solution with the help of magnetic stirrer
4. Different concentrations of hormonal supplements as required were added either in single or in different combination to this solution and were mixed thoroughly
5. Since each hormonal stock solution contained in 100 ml of solution, to make one liter of medium, addition of 1.0 ml/L and 1.5 ml/L NAA and 0.5 ml/L, 1.0 ml/L, 1.5 ml/L and 2.0 ml/L BAP singly was added to prepare 1 liter of medium
6. Later different combinations of these two hormones NAA and BAP respectively were used viz. (1.0+0.5), (1.0+1.0), (1.0+1.5), (1.0+2.0), (1.5+0.5), (1.5+1.0), (1.5+1.5), (1.5+2.0) mg/L
7. The whole mixture was then made up to 1 liter with further addition of double distilled water.

3.6. pH of the medium

pH of the medium was adjusted to 5.8 by pH meter with the addition of 1 N NaOH or 0.1 N HCl whichever was necessary.

3.7. Agar

The media was gelled with 8 g/L agar and the whole mixture was gently heated on microwave oven at 250 °C Temperature for 8-10 minutes.

3.8. Sterilization

3.8.1. Sterilization of culture media

Fixed volume of medium was dispensed into test tube. The test tubes were plugged with non- absorbent cotton. After dispensing the test tubes were covered with aluminum foil paper and marked with different codes with the help of a permanent glass marker to indicate specific hormonal supplement. Then the test tubes were autoclaved at 15 psi pressure at 121°C for 20 minutes. The medium was then transfer into the culture room and cooled at 24°C temperature before used. Marking is also necessary.

3.8.2. Sterilization of glassware and instruments

Glassware, culture vessels, beakers, petridishes, pipettes, slides, plastic caps, other instruments such as forceps, needles, scissor, spatula, surgical blades, brush, cotton, instrument stand and aluminum foil were sterilized in an autoclave at a temperature of 121°C for 20 minutes at 15psi pressure. Before this, all types of glassware instrument was washed properly by liquid detergent, cleaned with running tap water and finally washed with distilled water.

3.8.3. Sterilization of culture room and transfer area

In the beginning, the culture room was spray with formaldehyde and then the room was kept closed for one day. Then the room was cleaned through gently washing the floors walls and rakes with a detergent. This is followed by careful wiping them with 70% ethanol. This process of sterilization of culture room was repeated at regular intervals.

The transfer area was also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar air flow cabinet was usually sterilized by switching on the cabinet. The ultra violate ray kills the microbes inside the laminar airflow. It switches on 30 minutes before working in empty condition and for 20 minutes with all the instruments. The working surface was wiping with 70% ethanol, 30 minutes before starting the transfer work.

3.8.4. Precaution of ensure aseptic conditions

All inoculation and aseptic manipulations were carried out under laminar air flow cabinet. The cabinet was usually switched on with ultra violet light half an hour before use and wiped with 70% ethanol to reduce the chances of contamination. The instruments like scalpels, forceps, needles, surgical blades, scissor, pipettes, slides, plastic caps, spatula, brush, cotton etc. were presterilized by autoclaving and subsequent sterilization were done by dipping in 70% ethanol followed by flaming and cooling method inside the laminar flow cabinet. While not in use, the instruments were kept inside the laminar airflow cabinet into the instrument stand. Hands were also sterilized by 70% ethanol and wearing of hand gloves. It is also necessary to wear apron and mask to avoid contamination rate. Other required materials like distilled water, culture vessels, beakers, glass plates, petridishes etc. were sterilized in an autoclave following method of media sterilization. The neck of test tubes were flamed before open and also dipping with ethanol with the help of soaked cotton before closing it with the aluminum foil paper. Aseptic conditions were followed during each and every operation to avoid the contamination of cultures.

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3.3.15

3.9. Culture methods

The following culture methods were in work in the present investigation.

1. Explant culture
2. Subculture or transfer

3.9.1. Preparation of explants

The tuber sprout was first cut from the potato and washed thoroughly with double distilled water into the laminar airflow cabinet. For surface sterilization, sprouts were then sterilized with 70% (v/v) ethanol for one minute. After sterilization, the sprouts were then rinsed and washed with brush for three times with sterile distilled water into the petridish to remove the trace of alcohol. Afterwards the sprouts were transfer into another petridish and were again surface sterilized by immersing in 0.1% $HgCl_2$ solution supplement with three drops of Tween-20 mixtures and then finally rinsed and washed four times with sterilized distilled water. The surface sterilized disinfested sprouts were then cut into small segments and kept under sterilized distilled water into the sterilized petridishes to make the sprout alive. Now the explants were ready for inoculation.

3.9.2. Placement of the sprout

The surface sterilized sprouts were transferred aseptically to the test tube and flaming with lamp and covered with aluminum foil. Each and every test tube was labeled with a code that indicates hormonal dose, replication number and date of culture. Then the test tubes were incubating at 25± 2°C in dark condition under 24 hours dark periods into the growth chamber for 5-7 days or until callus induction. After initiation of callus, it was kept in light where it was 16/8 hours photoperiod. The observation of cultures was started from the 3rd day of inoculation and continued up to 28th days.

3.9.3. Inoculation of culture

The explants were prepared carefully under aseptic condition inside the laminar airflow cabinet. One explant was directly inoculated to each test tubes (25x10) containing 10 ml of MS medium supplemented with different hormone concentrations as per treatments. There were five replications for each treatment. The test tubes were covered and sealed with aluminum foil paper. The total operation was done in the laminar airflow cabinet on the clean bench in sterile condition.

3.9.4. Maintenance of calli

Callus initiated after 3-5 days of explant inoculation in the medium. The developed calli were also kept under 16 h photoperiod at 25±2 °C. The test tubes were checked daily to note the response and the development of contamination.

3.9.5. Culture incubation conditions

The prepared cultures were kept in a growth room on the shelves. All the cultures were kept at 25±2 °C illuminated with 1.83-m florescent tubes (4.83 ft C84 TDFL/ Philips). Those tubes broad sprectum of light, especially in the red wavelength. The room was illuminated 16 h daily with a light intensity of 3000 lux and monitored by using luxmeter. After four weeks of culture, callus tissues those which does not produce any shoot or root, were sub cultured at 21-28 days interval and finally transferred into the new medium. Plantlets developed from calli in this medium after 14- 21 days.

3.10. Subculture

3.10.1. Subculture of the callus for shoot regeneration

Four weeks after incubation of explants, the calli attained convenient size. Those which does not produce any root or shoot, were then remove aseptically from the test tubes on a sterilized petridishes, and callus was cut into small pieces with the help of sharp sterilized blade and place again on freshly prepared sterilized MS medium in a glass vials inside the laminar airflow cabinet for shoot, root initiation. The subcultured test tubes were then incubated at 25±°C with 16 h photoperiod. After shoot initiation, more light intensity was given for shoot elongation. The test tubes showing sign on contamination were discarded from the laboratory to reduce the rate of contamination. Repeated subculture was attended at regular interval of 21-25 days while incubated under the same temperature. The observations and data collections were noted regularly.

3.10.2. Subculture of the regenerated shoot for root initiation

The subculture calli contained proliferated and differentiated shoot and the microplants those which directly produce shoot from the calli was again need to subculture. When these shoots grew about 4-5 cm in length, were taken out from the flask, separated from each other and placed on a sterilized petridishes. Leaf, stem and internodal segments were cut aseptically into small pieces with the help of sharp sterilized blade or aseptic scissor and the small segments of stem were then used as explant. It was then again cultured in another culture vials with freshly prepared medium for root induction. Five segments of explants were directly inoculated into each vial containing 25 ml of MS medium.

3.11. Transfer of plantlets to *in vivo* condition

When the plantlets attained 30 days old with fully matured well developed shoots, leaves and roots, then *in vitro* rooted plants were removed from culture vials very carefully and aseptically with the help of fine forceps. Attached medium to roots were smoothly washed out with the help of soft brush in the running tap water to remove adhering gel. Then the plantlets were transplanted to plastic pots containing autoclaved garden soil, sand and cowdung in the ratio of 1:2:1 and covered with plastic paper. Plants were kept under culture room conditions for 15-20 days then transferred to net house and placed under shade until growth was observed.

Transplantation of the plantlets was done in the afternoon. Immediately after transplantation the plantlets were irrigated with a fine spray of water and the plantlets along with plastic trays were covered with transparent polythene bags to prevent desiccation. Initially plants are kept in high humidity and with low light intensity. The humidity is gradually decreased to the ambient level after 7-15 days and the light intensity is increased. It was irrigated regularly at an interval of 2 days.

3.12. Transfer of plantlets to the soil

After 7-10 days the plantlets were established and then the polythene bags were removed. Finally, the plantlets appeared too self-sustainable and then transfer to soil in open environment.

3.13. Experimental Factors

The experiment consisted of two factors.

A. Variety

B. Different concentrations of NAA and BAP.

3.13.1. Factor A: Experimental materials are BARI released three popular potato cultivars viz.

1. Granola (BARI Alu- 13)
2. Diamant (BARI Alu- 07)
3. Asterix (BARI Alu-25).

3.13.2. Factor B: Different concentrations of NAA and BAP.

3.14. Treatments

In vitro clonal propagation techniques were applied for callus induction and potato plantlet regeneration. Sprouted bud was used as explant. MS (Murashige and skoog, 1962) medium supplemented with NAA (α naphthalene acetic acid) and BAP (Benzyl amino purine), was used as treatment combination. NAA with the concentration of 0.0, 1.0 mg/L and 1.5 mg/L and BAP with the concentrations of 0.0, 0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L were used. Later different combinations of these two hormones were used viz. (1.0+0.5), (1.0+1.0), (1.0+1.5), (1.0+2.0), (1.5+0.5), (1.5+1.0), (1.5+1.5), (1.5+2.0) mg/L.

3.15. Experimental design

The experiment was laid in Completely Randomized design (CRD) having two factors (Variety and Treatment) with five replications.

3.16. Collection of data

To investigate the effect of different treatments of this experiment, the following parameters were recorded:

3.16.1. GA_3 application and sprout initiation in tuber

1. **Days to sprout initiation**: After spraying with variable concentrations of GA_3, starting date of spraying was recorded for each treatment and each variety. As soon as any sprout was initiated, record the data and count the number of days required to sprout initiation.

2. **Total sprouting time**: How much time the individual variety actually require for total sprouting.

3. **Sprout length (cm):** Maximum length of sprout was recorded in cm with the help of plastic scale.

4. **Number of sprout/potato:** Maximum number of sprout per potato was collected and recorded. It varied with different variety. The mean value was calculated using the following formula :

$$\overline{X} = \sum Xi / n$$

Where,

$\overline{X}$ = Mean of sprout/potato

$\sum$ = Summation

Xi = Number of sprout/potato

N = Number of observations/ replication

5. **Node/sprout:** The number of node per sprout was recorded.

3.16.2. *In vitro* callus induction

1. **Callus color:** The color of the callus was taken whether it is white, green, light green, deep green, brown, pink, yellow or in combination of those color by visual observation. And the color of the callus varies with the light intensity. It was white in color up to 5-7 days when it was kept in dark condition.

2. **Texture of callus**: Texture of the callus measured by either it is friable or non-friable/ compact for their physical characteristics.

3. **Days to callus induction:** Generally callus induction started after few days of explant incubation. Days to callus induction was recorded until callus was not induced from the explant. The mean value of the data provided the days to callus induction.

4. **Percentage of callus induction:** Percentage of callus induction were noted after 7-10 days of inoculation by using the following formula:

$$\text{Percentage of callus induction} = \frac{\text{No. of explants induced calli}}{\text{No. of induced calli}} \times 100$$

5. **Size of callus**: It was measured with a plastic scale set under test tube from base to apex of callus. Size of the callus was recorded at 7, 14, 21 28 days after inoculation (DAI) of callus. Callus length was measured horizontally and breadth was measured vertically. The formula (Thadavong *et al.*, 2002) used for estimating the size of callus is given below:

$$\text{Size of callus} = \frac{\text{Breadth} + \text{length}}{2}$$

6. **Fresh weight of callus (gm)**: Callus weights were recorded at 14 and 30 days in grams after inoculation (DAI) of explants with the help of electrical digital balance inside the laminar airflow with proper precaution. After that the callus was place in its previous place.

3.16.3. *In vitro* plant regeneration

To investigate the effect of different hormonal treatment of this experiment, the following parameters were recorded.

1. **Days to shoot initiation**: Shoot initiation started after 14-28 days of incubation of explants. The mean value of the data provided the days required for shoot initiation.

2. **Number of shoots/plantlet:** The number of shoot proliferated was recorded at 7, 14, 21 and 28 days after inoculation (DAI) and the number of shoots/explant was counted and mean was recorded. The mean value was calculated using the following formula:

   $\overline{X} = \sum Xi / n$

   Where,

   $\overline{X}$ = Mean of shoots/plant

   $\sum$ = Summation

   Xi = Number of shoots/plant

   N = Number of observations/replication

3. **Length of shoots/plantlet:** The length of shoots in cm was measured using a plastic scale in laminar airflow cabinet at an interval of 7, 14, 21 and 28 days after inoculation (DAI). The mean value of the data provided the shoot length.

4. **Number of leaves/plantlet:** Number of leaves/plantlet was recorded by visual observation at an interval of 7, 14, 21 and 28 days after inoculation (DAI). The mean value of the data provided the number of leaves/plantlet.

5. **Days to root initiation**: Root formation was initiated within 21-28 days. But in many cases within one week. The mean value of the data provided the days to root initiation.

6. **Number of roots/plantlet:** The number of roots/plantlet was recorded at an interval of 7, 14, 21and 28 days after inoculation (DAI) of explants inoculation and it was recorded and mean was calculated.

7. **Length of roots/plantlet:** The length of roots/plantlet was determined by using a plastic scale in side the laminar airflow cabinet by plotting the plant in a petridish. Length of root in cm was recorded at an interval of 7, 14, 21and 28 days after inoculation (DAI) of explants inoculation and it was recorded and mean was calculated.

3.16.4. Subculture of *in vitro* plantlet

1. Number of shoots/plantlet
2. Length of shoot/plantlet (cm)
3. Number of leaves/plantlet
4. Number of roots/plantlet
5. Length of roots/plantlet (cm)

3.17. Acclimatization

3.17.1. Transfer of plantlets from culture vessels to soil

During the *in vivo* acclimatization and the establishment of the previously regenerated and subcultured plantlets in to the soil, data were collected for the following parameter.

3.17.2. Survival rate (%) of plantlets

The survival rate of established plants was calculated based on the number of plants placed in the cubicles and the number of plants finally survived. The survival rate of plantlets established were calculated by using the following formula:

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

3.18. Statistical analysis

The data for the characters under present study were statistically analyzes where applicable. The experiment was conducted in growth chamber and arranged in Completely Randomized Design (CRD). Data were analyzed using MSTAT-C statistical package programme. The analysis of variance was performed and differences among the means were compared by the Least Significant Different test at 5% level of significance.

CHAPTER 4
RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

Three separate experiments were conducted to study the performance of different concentrations of GA_3 on sprout initiation of three BARI released cultivated potato varieties Granola, Diamant, Asterix, the effect of different hormone concentrations of NAA and BAP on *in vitro* callus induction, plant regeneration and their *in vivo* stablishment to the soil. The results obtained from these studies have been presented and discussed separately under different heading. Each of the parameter as influenced by varieties, treatments and their combinations were discussed. Some of the data have been given in Tables (1-19); while others shown in Plate (1-31) and Figure (1-3) for discussion, comprehension and understanding. Moreover, summarized analyses of variances in respect of parameters studies have been in Appendices (I-X).

4.1. Experiment 1: Sprouting potentiality of three BARI released potato under different concentrations of GA_3 treatment

The experiment was conducted to study the effect of five different concentrations (50, 100, 200, 300 and 400 ppm/L) of GA_3 on sprouting potentiality within short period of time on BARI released three potato varieties. The experiment was carried out under laboratory condition. The results are presented in Table 2.

4.1.1. Days to sprout initiation

The potato cultivars responded differently with different combination of GA_3 concentration. It was revealed that the variety Granola responded very quickly for sprouting. Days to sprout initiation was the minimum in 400 ppm/L of GA_3 application for all the three varieties (Table 2 and Plate 2). Within this concentration, Granola showed the minimum (3 days) days for sprouting and it was the maximum (5 days) in Asterix. Maximum days for sprout initiation was observed with 50 ppm of GA_3 in all the varieties, where Diamant took the maximum (12 days) and the minimum (9 days) was in Granola. It revealed that days to sprout initiation gradually decreased with the increased concentration of GA_3.

Table 2: Sprouting potentiality of three BARI released potato under different concentrations of GA_3 treatment

GA_3 Treatment (ppm/L)	Variety	Days to sprout initiation	Total sprouting time	Length of Sprout (cm)	Number of sprout/ potato	Node/ sprout
0	Granola	Sprouting was not occurred in off-season				
	Diamant					
	Asterix					
50	Granola	9	15	2-3	1	2
	Diamant	12	25	2-3	1	2
	Asterix	11	25	2-3	2	2
100	Granola	9	16	3-3.5	4	3
	Diamant	12	22	2-3	1	2
	Asterix	10	14	2-2.5	2	2
200	Granola	4	10	2-2.5	5	3
	Diamant	7	15	3	3	3
	Asterix	7	10	4	3	3
300	Granola	4	10	1.7-2	5	4
	Diamant	7	12	4	3	4
	Asterix	6	10	4	4	4
400	Granola	3	7	1-1.5	6	4
	Diamant	4	12	3-3.5	5	4
	Asterix	5	9	4	5	4

4.1.2. Total sprouting time

Total sprouting time was also the minimum in 400 ppm/L of GA_3 treatment Granola took only 7 days for total sprouting and it was the maximum (12 days) in Diamant.

4.1.3. Length of sprout

Average sprout length varied from 2-4 cm for all the treatments and varieties. Remarkable variation was not notice for this morphological trait among the experimental materials.

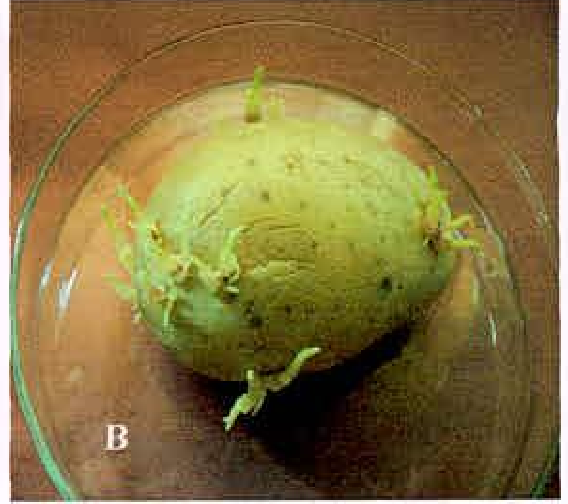
4.1.4. Number of sprout/potato

Number of sprout/potato was minimum 1 and it was maximum 6 in 400 ppm/L GA_3 application. It was observed that lower concentration of GA_3 produced minimum sprout for all the varieties under study and it increased generally with higher concentration.

4.1.5. Node/sprout

The maximum sprouting (4 node/sprout) was found in 400 ppm/L of GA_3 application. node/sprout ranged from 2-4 for all the treatments and varieties. No remarkable variation was responded for this trait.

The overall experimental findings revealed that the variety Granola was the most responsive to GA_3 application for sprouting. Higher concentration of GA_3 showed better performance to sprouting for all the materials under study. The treatment 400 ppm/L GA_3 concentration found the best for sprout initiation, because it required less time to get maximum number of sprout.



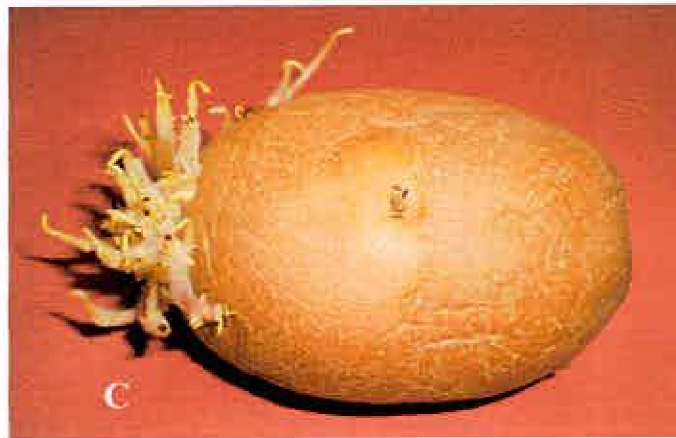


Plate 2. Maximum sprouting @ GA_3 400 ppm/L concentration. Sprouts of A. Granola, B. Diamant and C. Asterix

4.2. Experiment 2: Callus induction and plant regeneration of three potato varieties supplemented with different concentrations of NAA and BAP

4.2.1. Callus color and callus texture

Three potato varieties Granola, Diamant, Asterix were cultured on MS media supplemented with different concentrations of NAA and BAP. Color of calli were white from the beginning but DAI, calli were mostly change into green, light green, deep green, brown, pink and yellow. Most of the calli were non friable in texture.
The effect of NAA and BAP for callus induction are presented in Table 3-5. There was wide range of variation in days to callus induction. The result of the study have been presented and discussed under the following headings.

4.2.2. Days to callus induction

The results of major effect of varieties on days to callus induction have been presented in Table 3. The sprout segment from three potato varieties were used as explants and cultured on MS medium supplemented with different concentrations of NAA and BAP. The main effect of varieties revealed that there was significant difference on days to callus induction. The maximum days to callus induction (5.94 days) and the lowest (4.46 days) were noticed in Asterix and Diamant variety respectively.

There was significant influence of different hormone combinations and concentrations of NAA and BAP on days to callus induction. The maximum days (7.93 days) to callus induction was found at 1.0 mg/L NAA followed by 1.5 NAA mg/L (7.80 days) and the minimum (3.53 days) was observed in 1.0 mg/L NAA+1.5 mg/L BAP (Table 4 and Plate 3). This result is similar with Kollist *et al.* (1994). He found that low concentrations of auxin and high concentrations of cytokinin were effective for callus formation.

The combined effect of different potato varieties and hormone showed significant variation on days to callus induction (Appendix II). The maximum days (14 days) to callus induction was noticed in the treatment combination 1.5 mg/L NAA in variety Asterix. The minimum days (3 days) to callus induction was found in variety Granola with 1.0 mg/L NAA+1.5 mg/L BAP (Table 5). Auxin is an important media supplements for callus induction in culture. No callus was formed when explants of

different verities were cultured in fresh MS media without hormone and BAP alone (0.5,1.0,1.5 and 2.0 mg/L).

Table 3: Effect of different varieties on days to callus induction, size and fresh weight of callus at different days after inoculation

Variety	Days to callus induction	Size of callus (cm)				Fresh weight of callus (gm)	
		7 DAI	14 DAI	21 DAI	28 DAI	Initial weight (14 DAI)	Final weight (30 DAI)
Granola	4.56	0.42	0.72	1.07	1.61	0.89	1.91
Diamant	4.46	0.41	0.72	1.03	1.44	0.83	1.73
Asterix	5.94	0.39	0.64	1.00	1.42	0.63	1.30
SE±	0.164	0.007	0.017	0.024	0.029	0.025	0.047
Max	5.94	0.42	0.72	1.07	1.61	0.89	1.91
Min	4.46	0.39	0.64	1.00	1.42	0.63	1.30
LSD	0.416	0.144	0.067	0.033	0.136	0.111	0.220
Level of significance	**	NS	NS	NS	**	**	**

Table 4: Effect of different hormones on days to callus induction, size and fresh weight of callus at different days after inoculation

Hormone	Days to callus induction	Size of callus (cm)				Fresh weight of callus (gm)	
		7 DAI	14 DAI	21 DAI	28 DAI	Initial weight (14 DAI)	Final weight (30 DAI)
T1=Normal MS	-	-	-	-	-	-	-
T2=0.5BAP	-	-	-	-	-	-	-
T3=1.0BAP	-	-	-	-	-	-	-
T4=1.5BAP	-	-	-	-	-	-	-
T5=2.0BAP	-	-	-	-	-	-	-
T6= 1.0NAA	7.93	0.47	0.78	1.36	1.77	1.02	1.79
T7=1.0NAA+0.5BAP	3.67	0.37	0.59	1.03	1.31	0.92	1.98
T8=1.0NAA+1.0BAP	4.20	0.38	0.64	0.86	1.22	0.72	1.63
T9=1.0NAA+1.5BAP	3.53	0.34	0.54	0.71	1.14	0.65	1.15
T10=1.0NAA+2.0BAP	5.73	0.37	0.62	0.86	1.31	0.53	1.46
T11= 1.5NAA	7.80	0.49	0.86	1.36	1.94	0.87	2.08
T12=1.5NAA+0.5BAP	3.93	0.38	0.64	0.97	1.41	0.57	1.25
T13=1.5NAA+1.0BAP	4.13	0.44	0.81	1.11	1.76	1.01	1.95
T14=1.5NAA+1.5BAP	4.40	0.40	0.76	1.03	1.53	0.68	1.46
T15=1.5NAA+2.0BAP	4.53	0.40	0.70	1.03	1.51	0.87	1.71
SE±	0.164	0.007	0.017	0.024	0.029	0.025	0.047
Max	7.93	0.49	0.86	1.36	1.94	1.02	2.08
Min	3.53	0.34	0.54	0.71	1.14	0.53	1.15
LSD	0.759	0.264	0.123	0.060	0.248	0.203	0.401
Level of significance	**	**	**	**	**	**	**

Table 5: Combined effect of different varieties and different hormones on days to callus induction, size and fresh weight of callus at different days after inoculation

Variety	Hormone	Days to callus induction	Size of callus (cm)				Fresh weight of callus (gm)	
			7 DAI	14 DAI	21 DAI	28 DAI	Initial weight (14 days)	Final weight (30 days)
Granola	T1=Normal MS	-	-	-	-	-	-	-
	T2=0.5BAP	-	-	-	-	-	-	-
	T3=1.0BAP	-	-	-	-	-	-	-
	T4=1.5BAP	-	-	-	-	-	-	-
	T5=2.0BAP	-	-	-	-	-	-	-
	T6= 1.0NAA	7.00	0.42	0.81	1.34	1.76	1.36	2.37
	T7=1.0NAA+0.5BAP	3.80	0.29	0.36	0.86	0.95	0.31	0.64
	T8=1.0NAA+1.0BAP	3.80	0.41	0.72	0.97	1.39	0.99	2.21
	T9=1.0NAA+1.5BAP	3.00	0.38	0.66	0.90	1.16	0.87	1.90
	T10=1.0NAA+2.0BAP	5.60	0.40	0.75	0.89	1.54	0.64	1.32
	T11= 1.5NAA	4.40	0.66	1.02	1.42	1.90	1.04	2.12
	T12=1.5NAA+0.5BAP	4.80	0.38	0.51	0.77	1.53	0.64	1.43
	T13=1.5NAA+1.0BAP	4.80	0.38	0.76	1.22	1.98	0.62	1.44
	T14=1.5NAA+1.5BAP	4.00	0.49	0.99	1.26	1.65	0.78	1.55
	T15=1.5NAA+2.0BAP	4.40	0.36	0.63	1.03	1.74	0.73	1.49
Diamant	T1=Normal MS	-	-	-	-	-	-	-
	T2=0.5BAP	-	-	-	-	-	-	-
	T3=1.0BAP	-	-	-	-	-	-	-
	T4=1.5BAP	-	-	-	-	-	-	-
	T5=2.0BAP	-	-	-	-	-	-	-
	T6= 1.0NAA	5.40	0.54	0.92	1.45	1.56	0.73	1.06
	T7=1.0NAA+0.5BAP	3.40	0.41	0.69	1.07	1.45	0.85	1.92
	T8=1.0NAA+1.0BAP	4.00	0.32	0.51	0.72	1.49	0.65	1.47
	T9=1.0NAA+1.5BAP	4.20	0.30	0.42	0.53	0.98	0.52	1.10
	T10=1.0NAA+2.0BAP	6.20	0.34	0.54	0.66	1.17	0.37	0.79
	T11= 1.5NAA	5.00	0.50	0.90	1.75	2.10	0.67	2.54
	T12=1.5NAA+0.5BAP	3.40	0.38	0.70	0.96	1.50	0.34	0.89
	T13=1.5NAA+1.0BAP	4.00	0.50	1.02	1.16	1.76	1.31	2.43
	T14=1.5NAA+1.5BAP	4.80	0.36	0.64	0.88	1.40	0.52	1.27
	T15=1.5NAA+2.0BAP	4.20	0.43	0.83	1.07	1.33	0.67	1.35
Asterix	T1=Normal MS	-	-	-	-	-	-	-
	T2=0.5BAP	-	-	-	-	-	-	-
	T3=1.0BAP	-	-	-	-	-	-	-
	T4=1.5BAP	-	-	-	-	-	-	-
	T5=2.0BAP	-	-	-	-	-	-	-
	T6= 1.0NAA	11.40	0.46	0.61	1.30	1.99	0.98	1.92
	T7=1.0NAA+0.5BAP	3.40	0.41	0.73	1.15	1.49	1.25	1.54
	T8=1.0NAA+1.0BAP	4.80	0.41	0.69	0.88	1.33	0.82	1.78
	T9=1.0NAA+1.5BAP	3.80	0.35	0.54	0.69	1.29	0.57	1.39
	T10=1.0NAA+2.0BAP	5.40	0.37	0.57	1.02	1.23	0.58	1.33
	T11= 1.5NAA	14.00	0.30	0.50	0.90	1.83	0.90	2.19
	T12=1.5NAA+0.5BAP	3.60	0.38	0.70	1.19	1.21	0.77	1.67
	T13=1.5NAA+1.0BAP	3.60	0.43	0.79	0.94	1.55	1.11	2.37
	T14=1.5NAA+1.5BAP	4.40	0.36	0.66	0.96	1.53	0.75	1.57
	T15=1.5NAA+2.0BAP	5.00	0.41	0.65	0.99	1.46	1.20	2.31
	SE±	0.164	0.007	0.017	0.024	0.029	0.025	0.047
	Max	14.00	0.66	1.02	1.75	2.10	1.36	2.54
	Min	3.00	0.29	0.36	0.53	0.95	0.31	0.64
	LSD	1.315	0.457	0.213	0.105	0.430	0.352	0.695
	Level of significance	**	**	**	**	NS	**	**

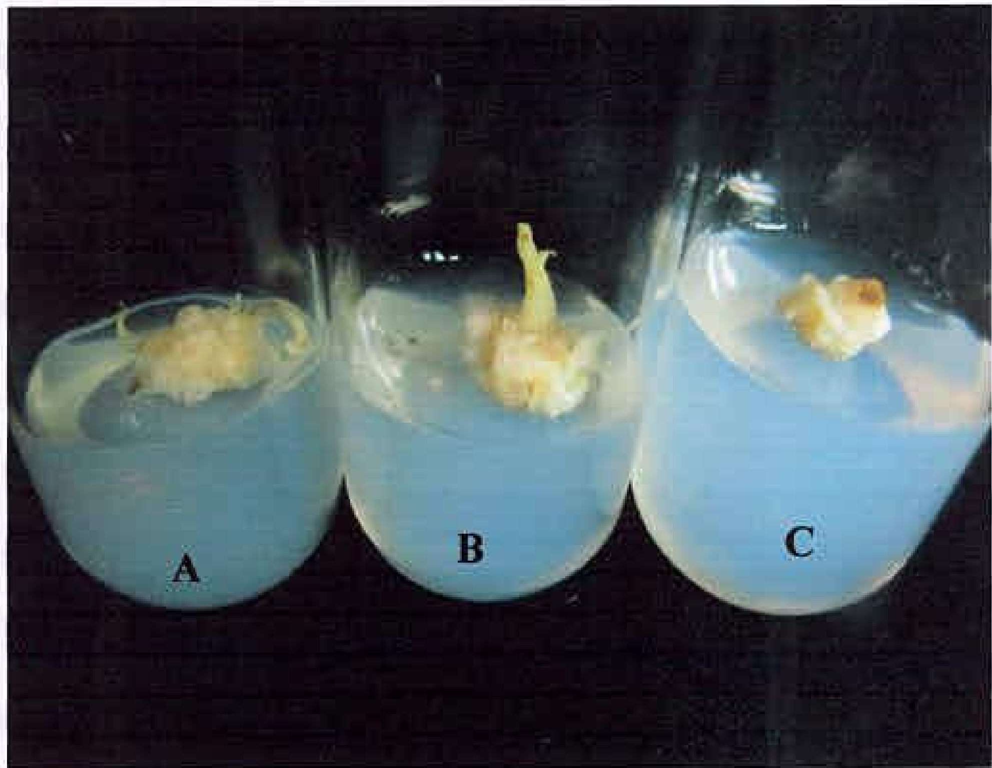


Plate 3. Callus induction at 7 DAI on MS media supplemented with 1.0 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

4.2.3. Percentage of callus induction

Percentage of callus induction varied among the varieties used in the present study. NAA with the concentration of 1.0 mg/L (T6) and 1.5 mg/L (T11) alone induced callus and also in combination with BAP, these two hormones viz. 1.0 mg/L NAA+0.5 mg/L BAP (T7), 1.0mg/L NAA+1.0mg/L BAP (T8), 1.0 mg/L NAA+1.5 mg/L BAP(T9), 1.0 mg/L NAA+2.0 mg/L BAP (T10), 1.5 mg/L NAA+0.5 mg/L BAP(T12), 1.5 mg/L NAA+1.0 mg/L BAP(T13), 1.5 mg/L NAA+1.5 mg/L BAP (T14), 1.5 mg/L NAA+2.0 mg/L BAP(T15) were also induced callus. MS media (T1) without hormone and BAP 0.5 mg/L (T2) 1.0 mg/L (T3), 1.5 mg/L (T4) and 2.0 mg/L(T5) alone did not induce callus.

In the case of Granola no callus was formed in T1 (Normal MS), T2 (0.5 mg/L BAP), T3 (1.0 mg/L BAP), T4 (1.5 mg/L BAP) and T5 (2.0 mg/L BAP). Maximum percentage (100%) of callus was induced in treatment T7 (1.0 mg/L NAA+0.5 mg/L BAP), T9 (1.0 mg/L NAA+1.5 mg/L BAP) and T13 (1.5 mg/L NAA+1.0 mg/L BAP) and minimum percentage (80%) of callus was induced in T10 (1.0 mg/L NAA+2.0 mg/L BAP) and T12 (1.5 mg/L NAA+0.5 mg/L BAP) (Figure 1).

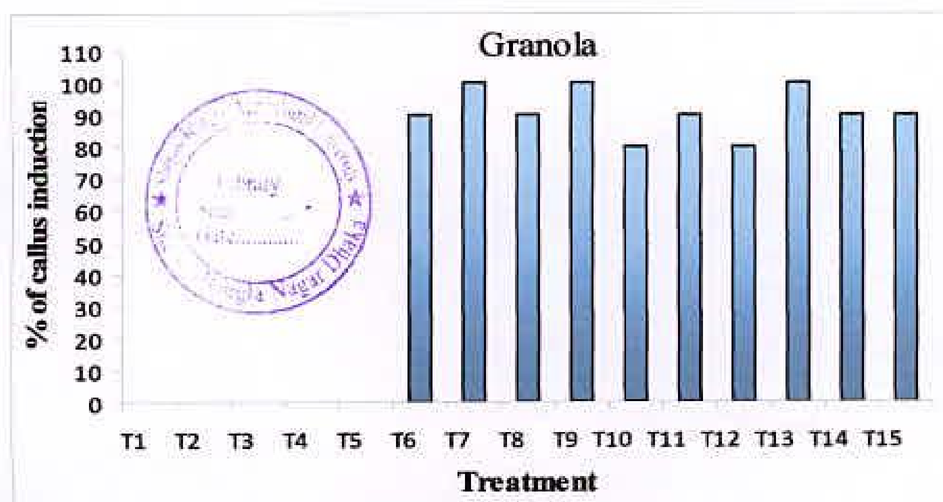


Figure 1: Effect of different hormones on percentage of callus induction of Granola

In the case of Diamant, no callus was induced in T1 (Normal MS), T2 (0.5 mg/L BAP), T3 (1.0 mg/L BAP), T4 (1.5 mg/L BAP) and T5 (2.0 mg/L BAP). Maximum percentage (100%) of callus was formed in T7 (1.0 mg/L NAA+0.5 mg/L BAP) and T12 (1.5 mg/L NAA+0.5 mg/L BAP) and it was minimum (60%) in T10 (1.0 mg/L NAA+2.0 mg/L BAP) (Figure 2).

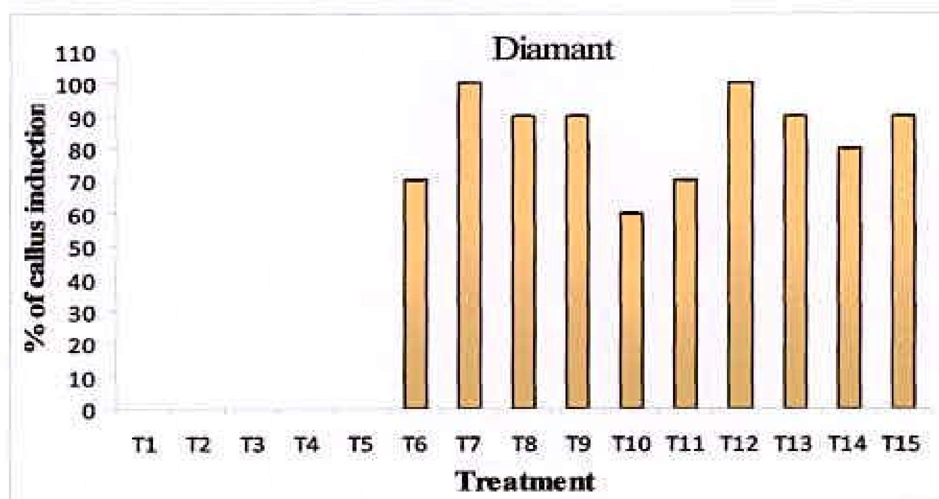


Figure 2: Effect of different hormones on percentage of callus induction of Diamant.

When the variety Asterix took under consideration, it was revealed that no callus was induced in T1 (Normal MS), T2 (0.5 mg/L BAP), T3 (1.0 mg/L BAP), T4 (1.5 mg/L BAP) and T5 (2.0 mg/L BAP). The maximum percentage of callus induced in T12 (1.5 mg/L NAA+0.5 mg/L BAP) and T13 (1.5 mg/L NAA+1.0 mg/L BAP) and it was minimum (50%) in T6 (1.0 mg/L NAA) (Figure 3).

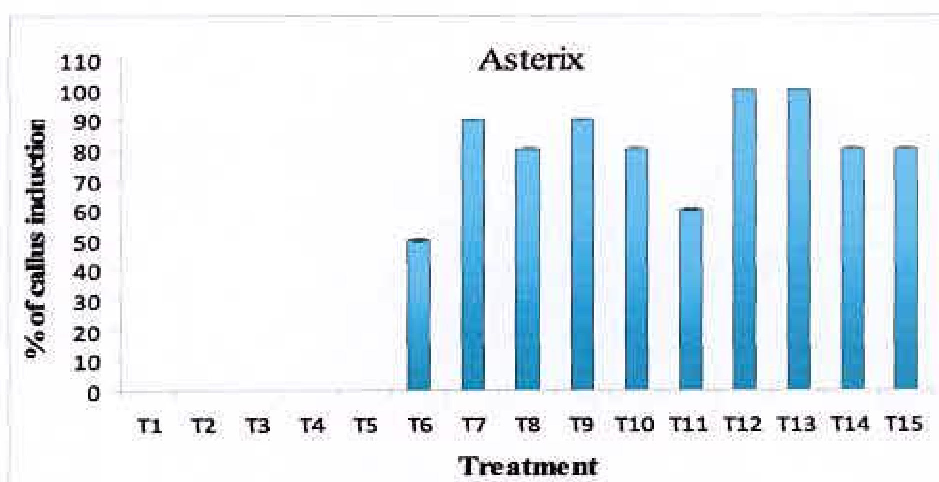


Figure 3: Effect of different hormones on percentage of callus induction of Asterix

4.2.4. Size of callus

The size of callus was recorded after 7, 14, 21 and 28 days of cultured on MS media containing different concentrations of NAA and BAP. The results have been presented in Table 3-5 and Plate 4-7.

The main effect of varieties showed non significance variation on size of callus at different DAI (Appendix IV) (Table 3). The highest size of callus was found in the variety Granola (0.42, 0.72, 1.07 and 1.61 cm at 7, 14, 21 and 28 DAI, respectively). The size of the callus increased gradually with advancement of DAI in this variety. The minimum size of callus was found in the variety Asterix (0.39, 0.64, 1.0 and 1.42 cm at 7, 14, 21 and 28 DAI, respectively).

The main effect of different concentrations of NAA and BAP showed significant variation on size of callus at different DAI. At 7, 14, 21 and 28 DAI, the hormone concentration of 1.5 mg/L NAA showed the highest size of callus (0.49, 0.86, 1.36 and 1.94 cm respectively at 7, 14, 21 and 28 DAI) and the lowest size of callus (0.34, 0.54, 0.71 and 1.14 cm, respectively) was observed at the hormonal combination 1.0 mg/L NAA +1.5 mg/L BAP (Table 4). This result was supported by Isaacs (1979) who stated that callus growth enhanced in medium supplemented with NAA and 2, 4-D.

The combined effect of varieties and different concentrations of hormones showed significant variation on size of callus at different DAI (Table 5). But in 28 DAI it showed non significant variation. At 7, 14, DAI the highest callus size (0.66 and 1.02 cm respectively) was observed at 1.5 mg/L NAA with the variety Granola and the lowest size of callus (0.29 and 0.36 cm, respectively) was found at 1.0 mg/L NAA+0.5 mg/L BAP in the same variety. At 28 DAI, the highest size of callus (2.10 cm) was found at 1.5 mg/L NAA with the variety Diamant and the lowest size of callus (0.95 cm) was observed at 1.0 mg/L NAA +0.5 mg/L BAP in the variety Granola.

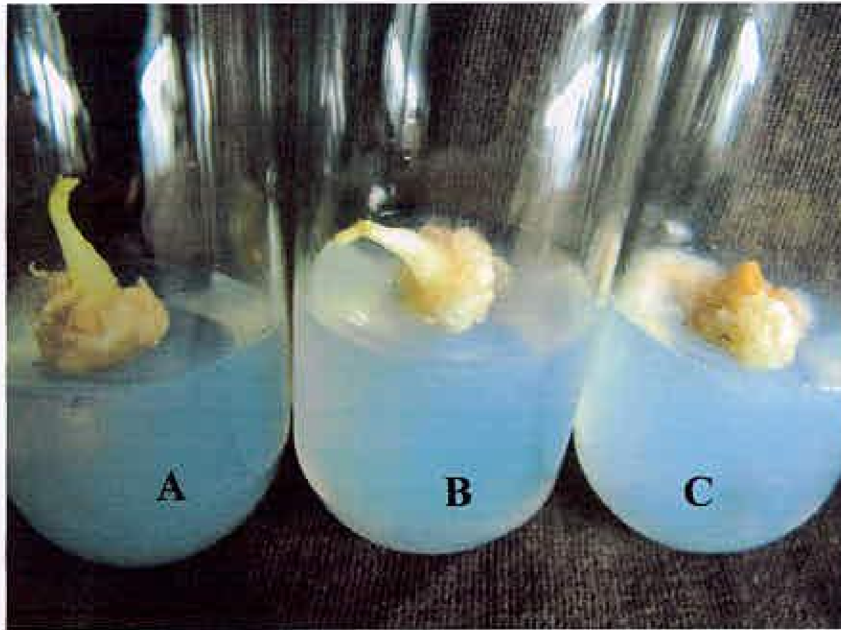


Plate 4. Maximum size of callus at 7 DAI on MS media supplemented with 1.5 mg/L NAA in A. Granola, B. Diamant and C. Asterix

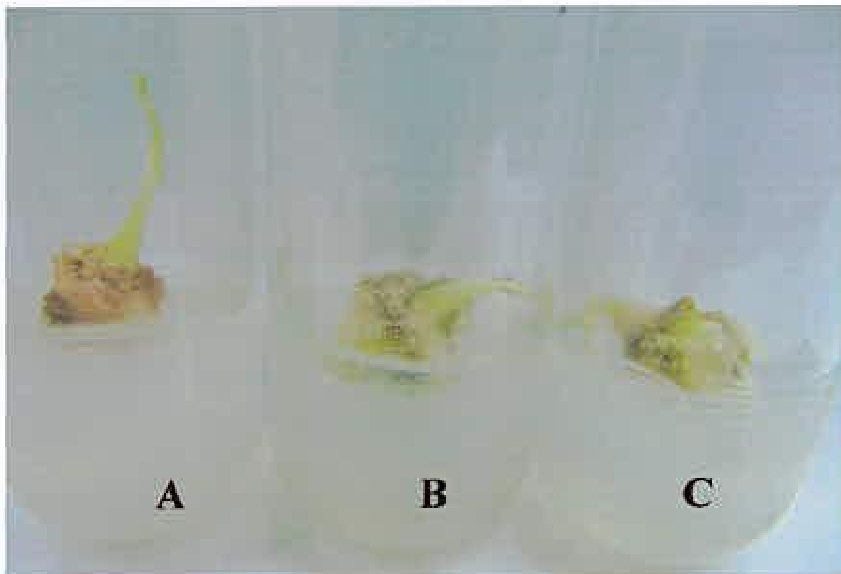


Plate 5. Maximum size of callus at 14 DAI on MS media supplemented with 1.5 mg/L NAA in A. Granola, B. Diamant and C. Asterix

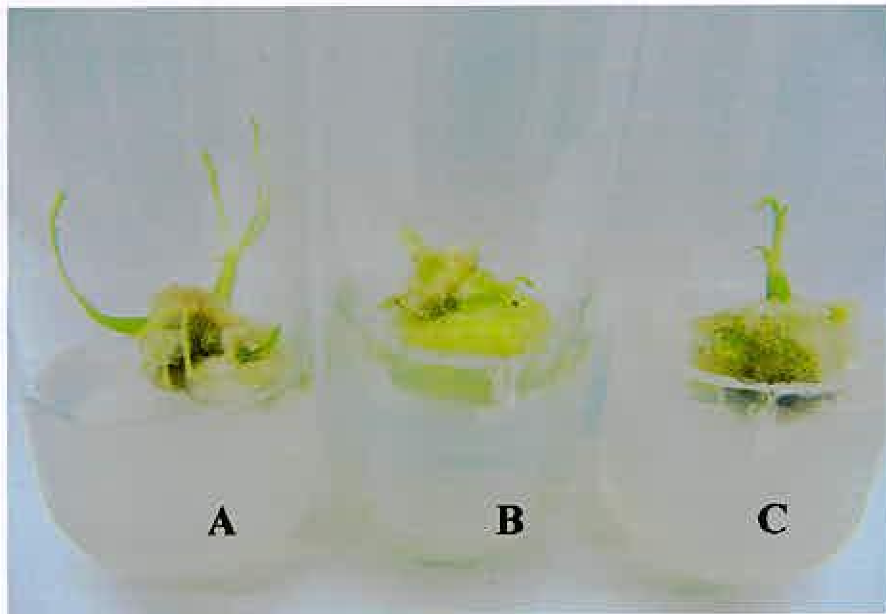


Plate 6. Maximum size of callus at 21 DAI on MS media supplemented with 1.5 mg/L NAA in A. Granola, B. Diamant and C. Asterix

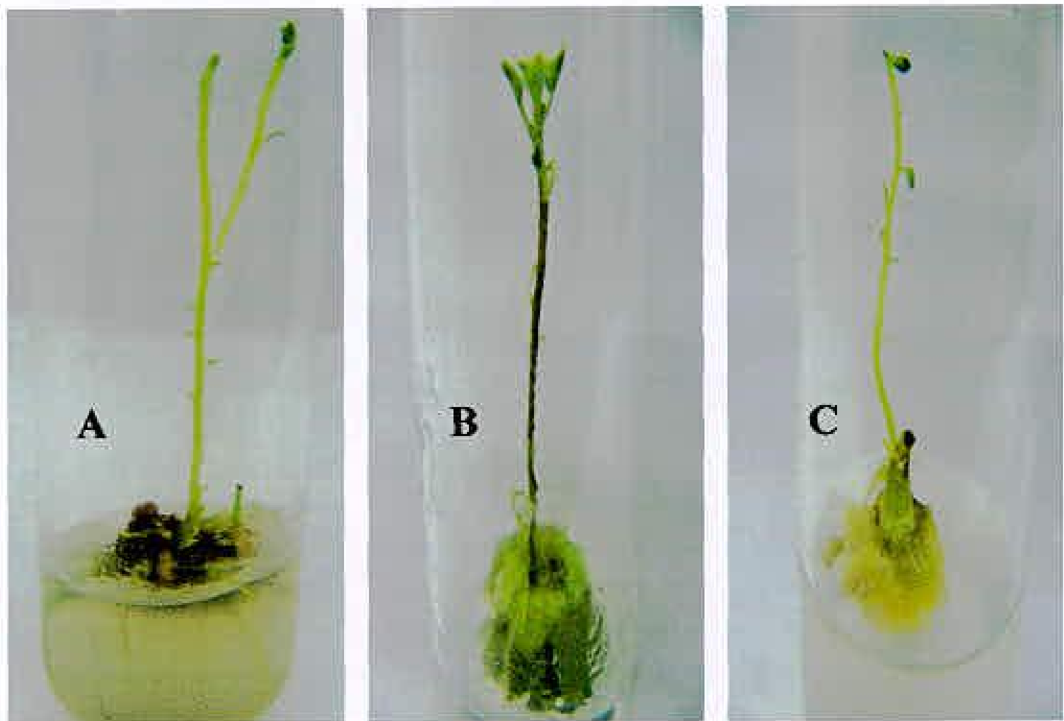


Plate 7. Maximum size of callus at 28 DAI on MS media supplemented with 1.5 mg/L NAA in A. Granola, B. Diamant and C. Asterix

4.2.5. Fresh weight of callus

There was significant difference in initial and final weight of callus among the varieties. The maximum weight of callus was observed in cv. Granola (0.89 gm and 1.91 gm at 14 DAI and 30 DAI respectively) whereas the minimum weight of callus was recorded in variety Asterix (0.63 gm and 1.30 gm at 14 DAI and 30 DAI, respectively) (Table 3).

The weight of callus was observed to be increased significantly due to different level of hormones (Table 4). The maximum weight of callus (1.02 gm) at 14 DAI was found in the treatment 1.0 mg/L NAA whereas the minimum weight of callus was (0.53gm) at treatment combination 1.0 mg/L NAA+2.0 mg/L BAP. At 30 DAI the maximum weight was recorded (2.08 gm) at 1.5 mg/L NAA. On the other hand, 1.0 mg/L NAA+1.5 mg/L BAP produced the minimum weight of callus (1.15 gm) (Table 4). This result is similar with Gynheung *et al.* (1986) and partially aggre the result of Fiegert *et al.* (2000).

There was significant interaction effect between the varieties and hormone concentrations on callus weight at 14 and 30 DAI. The highest weight of callus (1.36 gm at 14 DAI) was found in the variety Granola with the treatment combination 1.0 mg/L NAA and the lowest weight (0.31 gm and 0.64 gm at 14 DAI and 30 DAI, respectively) in the variety Granola with the treatment combination 1.0 mg/L NAA+0.5 mg/L BAP. Whereas at 30 DAI the maximum weight (2.54 gm) was observed in the variety Diamant with the treatment combination 1.5 mg/L NAA (Table 5). Fomenko *et al.* (1998) also showed that different genotypes reacted differently to the growth regulators in terms of callus formation.

4.2.6. 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 NAA and BAP. The results have been presented in Table 6-8 and Plate 8.

Potato varieties differed significantly in days to shoot initiation. In 7 DAI it showed non significant variation among the varieties. The maximum days to shoot initiation was observed in variety Asterix (15.09 days) whereas the minimum days was recorded in variety Granola (13.17days) (Table 6). Ao and Liu (1998) observed the varietal differences of shoot regeneration in potato. Hamdi *et al.* (1998) also reported the similar results.

Table 6: Effect of different varieties on days to shoot initiation and number of shoots/plantlet at different days after inoculation

Variety	Days to shoot initiation	Number of shoots/plantlet			
		7 DAI	14 DAI	21 DAI	28 DAI
Granola	13.17	0.84	1.59	2.13	3.00
Diamant	13.63	0.52	0.92	1.52	2.12
Asterix	15.09	0.44	0.91	1.12	1.91
SE±	0.567	0.058	0.081	0.089	0.120
Max	15.09	0.84	1.59	2.13	3.00
Min	13.17	0.44	0.91	1.12	1.91
LSD	0.995	0.128	0.198	0.217	0.366
Level of significance	**	**	**	**	**

There was also significant influence of different concentrations of NAA and BAP on days to shoot initiation. The maximum days to shoot initiation was recorded on 1.5 mg/L NAA (23.47 days) and 1.5 mg/L NAA+1.5 mg/L BAP required minimum days (6.87 Days) to shoot initiation (Table 7).

Table 7: Effect of different hormones on days to shoot initiation and number of shoots/plantlet at different days after inoculation

Hormone	Days to shoot initiation	Number of shoots/plantlet			
		7 DAI	14 DAI	21 DAI	28 DAI
T1=Normal MS	7.00	1.00	1.27	1.80	2.40
T2=0.5BAP	7.00	1.67	2.67	3.00	4.07
T3=1.0BAP	7.00	1.67	2.27	2.53	3.73
T4=1.5BAP	11.33	0.40	1.00	1.20	1.60
T5=2.0BAP	7.00	1.27	2.13	2.87	3.73
T6= 1.0NAA	22.27	0.00	0.20	0.60	1.27
T7=1.0NAA+0.5BAP	18.73	0.33	0.67	1.33	1.87
T8=1.0NAA+1.0BAP	22.93	0.00	0.27	0.93	1.67
T9=1.0NAA+1.5BAP	16.07	0.00	0.07	0.86	1.73
T10=1.0NAA+2.0BAP	10.47	0.27	0.60	0.93	1.13
T11= 1.5NAA	23.47	0.00	0.07	0.40	0.87
T12=1.5NAA+0.5BAP	9.33	0.67	1.67	2.33	2.67
T13=1.5NAA+1.0BAP	22.67	0.00	0.40	0.53	1.53
T14=1.5NAA+1.5BAP	6.87	1.73	3.00	3.40	4.87
T15=1.5NAA+2.0BAP	17.33	0.00	0.80	1.13	2.00
SE±	0.567	0.058	0.081	0.089	0.120
Max	23.47	1.73	3.00	3.40	4.87
Min	6.87	0.27	0.07	0.40	0.87
LSD	2.226	0.286	0.442	0.485	0.818
Level of significance	**	**	**	**	**

The combined effect of varieties and hormonal combinations on days to shoot initiation has been presented in the Table 8. Here the maximum days to shoot initiation was found in variety Asterix (29.60 days) with 1.0 mg/L NAA+1.0 mg/L BAP and the minimum days to shoot initiation in variety Granola (5.40 days) at 1.5 mg/L NAA+1.5 mg/L BAP.

Table 8: Combined effect of different varieties and different hormones on shoot initiation and number of shoots/plantlet at different days after inoculation

Variety	Hormone	Days to shoot initiation	Number of shoots/plantlet			
			7 DAI	14 DAI	21 DAI	28 DAI
Granola	T1=Normal MS	7.00	1.00	1.00	1.60	2.60
	T2=0.5BAP	7.00	1.60	2.60	3.00	4.00
	T3=1.0BAP	7.00	3.00	3.60	4.60	5.80
	T2=1.5BAP	7.00	1.0	2.0	3.0	4.0
	T5=2.0BAP	7.00	1.40	3.00	3.40	4.60
	T6= 1.0NAA	13.00	0.20	1.00	1.00	1.60
	T7=1.0NAA+0.5BAP	14.00	0.80	1.60	2.20	2.60
	T8=1.0NAA+1.0BAP	19.60	0.00	0.40	1.60	1.80
	T9=1.0NAA+1.5BAP	21.00	0.00	0.20	1.00	1.60
	T10=1.0NAA+2.0BAP	17.40	0.40	1.20	1.80	1.80
	T11= 1.5NAA	15.40	0.00	1.00	1.60	1.80
	T12=1.5NAA+0.5BAP	25.20	0.00	0.00	0.80	2.40
	T13=1.5NAA+1.0BAP	14.00	0.00	1.20	1.20	2.20
	T14=1.5NAA+1.5BAP	5.40	3.20	4.00	4.80	7.00
	T15=1.5NAA+2.0BAP	17.60	0.00	0.60	0.80	1.80
Diamant	T15=Normal MS	7.00	1.00	1.00	1.40	1.40
	T11=0.5BAP	7.00	1.00	3.00	3.40	6.40
	T12=1.0BAP	7.00	1.00	1.80	1.80	2.20
	T13=1.5BAP	7.00	1.00	1.80	2.60	3.20
	T14=2.0BAP	7.00	1.40	1.40	2.80	3.20
	T9= 1.0NAA	14.00	0.00	1.00	1.60	1.60
	T1=1.0NAA+0.5BAP	16.80	0.20	0.40	1.40	2.00
	T2=1.0NAA+1.0BAP	19.60	0.00	0.40	1.20	1.40
	T3=1.0NAA+1.5BAP	7.00	1.00	2.00	3.00	3.00
	T4=1.0NAA+2.0BAP	0.00	0.00	0.00	0.00	0.00
	T10= 1.5NAA	27.20	0.00	0.00	0.20	1.00
	T5=1.5NAA+0.5BAP	21.20	0.00	0.00	1.00	1.40
	T6=1.5NAA+1.0BAP	26.60	0.00	0.00	0.20	1.40
	T7=1.5NAA+1.5BAP	14.20	0.00	1.00	1.40	2.00
	T8=1.5NAA+2.0BAP	22.80	0.00	0.00	0.80	1.00
Asterix	T1=Normal MS	8.20	1.00	1.80	2.40	3.20
	T2=0.5BAP	7.00	2.40	3.00	3.00	3.20
	T3=1.0BAP	7.00	1.00	1.00	1.00	2.60
	T4=1.5BAP	7.00	1.00	2.60	3.00	3.60
	T5=2.0BAP	7.00	1.00	2.00	2.40	3.40
	T6= 1.0NAA	7.00	1.00	1.00	1.00	1.60
	T7=1.0NAA+0.5BAP	25.40	0.00	0.00	0.40	1.00
	T8=1.0NAA+1.0BAP	29.60	0.00	0.00	1.00	1.80
	T9=1.0NAA+1.5BAP	0.00	0.00	0.00	0.00	0.00
	T10=1.0NAA+2.0BAP	14.00	0.40	0.60	1.00	1.60
	T11= 1.5NAA	14.00	0.00	1.00	1.00	1.00
	T12=1.5NAA+0.5BAP	24.00	0.00	0.20	0.75	1.40
	T13=1.5NAA+1.0BAP	27.40	0.00	0.00	0.20	1.00
	T14=1.5NAA+1.5BAP	22.40	0.00	0.40	0.40	2.20
	T15=1.5NAA+2.0BAP	26.40	0.00	0.00	0.20	1.00
	SE±	0.567	0.058	0.081	0.089	0.120
	Max	29.60	3.20	4.00	4.80	7.00
	Min	5.40	0.20	0.20	0.20	1.00
	LSD	3.855	0.496	0.765	0.840	1.417
	Level of significance	**	**	**	**	**

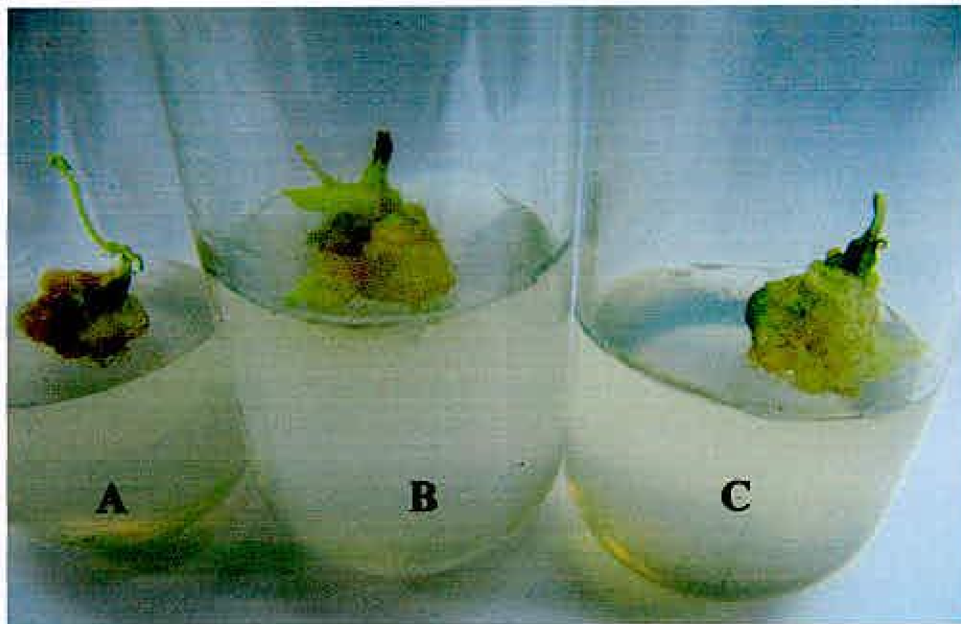


Plate 8. Initiated shoot at 7 DAI on MS media supplemented with 1.5 mg/L NAA+1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

4.2.7. Number of shoots/plantlet

The main effect of potato varieties exhibit significant variation in respect of number of shoots/plantlet at 7, 14, 21 and 28 DAI (Appendix VI). The variety Granola produced the highest number of shoots (0.84, 1.59, 2.13 and 3.00 shoots/plantlet at 7, 14, 21 and 28 DAI ,respectively) whereas the variety Asterix produced the lowest number of shoots (0.44, 0.91, 1.12 and 1.91 shoots/plantlet at 7, 14, 21 and 28 DAI, respectively) (Table 6).

The main effect of different concentration of NAA and BAP gave the significant variation in respect to number of shoots/plantlet (Appendix VI, Table 7, Plate 9-12) at 7, 14, 21 and 28 DAI. It was observed that at 7, 14, 21 and 28 days of culture, the maximum number of shoots (1.73, 3.00, 3.40 and 4.87 shoots/plantlet at 7, 14, 21 and 28 DAI, respectively) was produced by 1.5 mg/L NAA+1.5 mg/L BAP. At 21 and 28 days of culture, the minimum shoots/plantlet (0.40 and 0.87 shoots/plantlet, respectively) was observed in the treatment combination 1.5 mg/L NAA.

Combined effect of varieties and different concentrations of hormones showed significant variation at different days on number of shoots/plantlet. The highest number of shoot /plantlet (3.20, 4.00, 4.80 and 7.00 shoots/plantlet at 7, 14, 21 and 28 DAI, respectively) was recorded in variety Granola at 1.5 mg/L NAA+1.5 mg/L BAP and the lowest number of shoots/plantlet (1.0 shoots/plantlet at 28 DAI) was generated in variety Diamant in treatment combination 1.5 mg/L NAA and 1.5 mg/L NAA+2.0 mg/L BAP (Table 8). Similar result was also found in the variety Asterix with treatment combination 1.5 mg/L NAA, 1.0 mg/L NAA+0.5 mg/L BAP and 1.5 mg/L NAA+2.0 mg/L BAP.

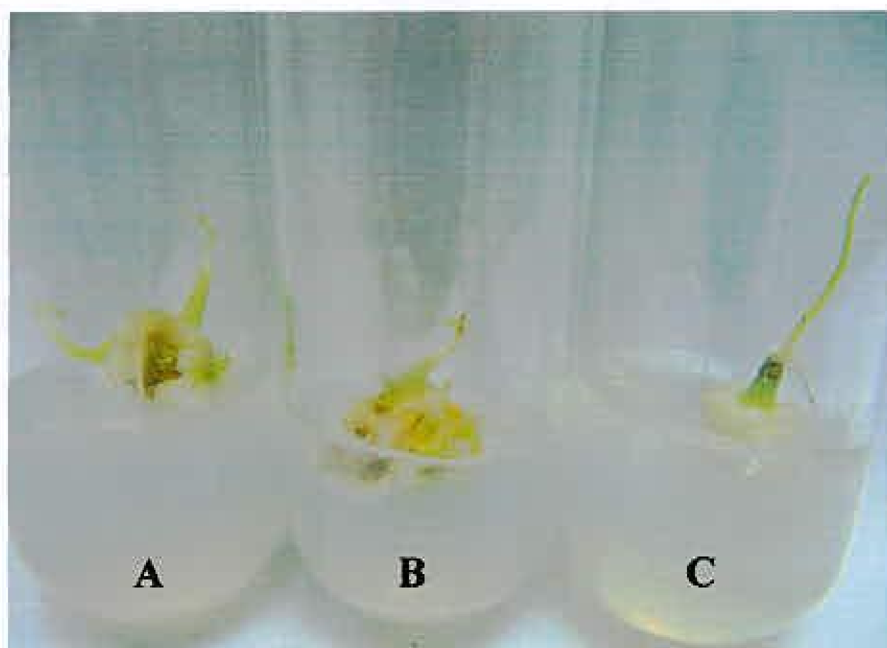


Plate 9. Maximum number of shoot initiated at 7 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

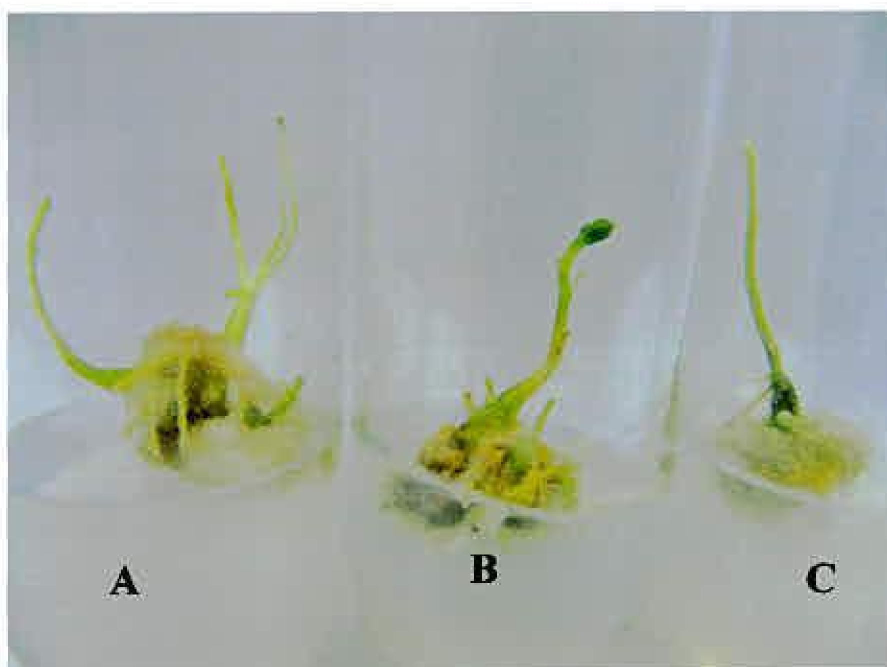


Plate 10. Maximum number of shoot initiated at 14 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

Plate 11. Maximum number of shoot initiated at 21 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

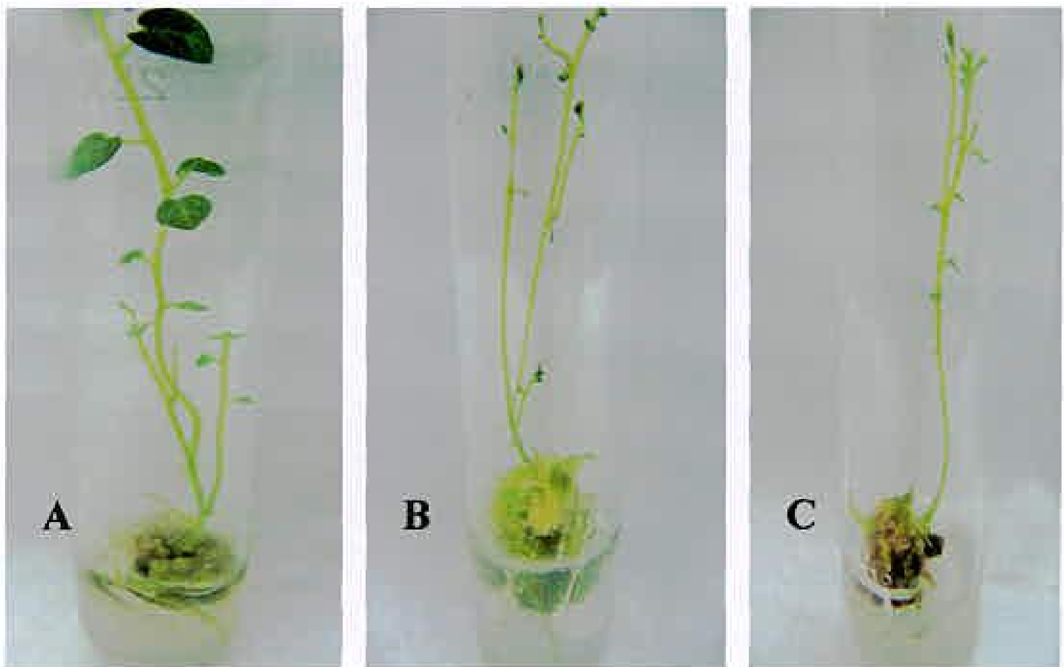


Plate 12. Maximum number of shoot initiated 28 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

4.2.8. Length of shoots/plantlet

Length of shoots/plantlet was significantly influenced by the three varieties of potato (Appendix VII). Length of shoot of different varieties was significantly increased at 7, 14, 21 and 28 DAI. At 7 days the highest length of shoot was found in the variety Asterix (1.03 cm) and the lowest length was found in the variety Diamant (0.96 cm). At 14, 21 and 28 days the highest length of shoot (2.38, 3.88 and 6.76 cm at 14, 21 and 28 days, respectively) was obtained from the variety Granola and the lowest length (1.76, 2.73 and 5.68 cm at 14, 21 and 28 days, respectively) was found from the variety Asterix (Table 9).

The main effect of different concentration of hormones on length of shoots/plantlet has been presented in Table 10 and Plate 13-16. There was a significant variation among the treatments. Among the treatments, it was observed that length of shoot increased gradually with the advancement of time. At 7, 14, 21 and 28 DAI, the longest shoot (2.88, 4.23, 5.83 and 10.13 cm at 7, 14, 21 and 28, respectively) was obtained from the treatment combination 1.5 mg/L NAA+1.5 mg/L BAP. At 28 DAI the shortest shoot (2.14 cm) was obtained from 1.0 mg/L NAA+2.0 mg/L BAP.

The combined effect of different varieties and different concentrations of NAA and BAP also showed significant differences (Appendix VII). At 7 DAI, the longest shoot (3.26cm) was produced by the treatment combination 1.0 mg/L BAP with variety Asterix and the shortest length (0.14 cm) from the treatment combination 1.0 mg/L NAA+2.0 mg/L BAP with the variety Granola. At 28 DAI, the highest length (10.72 cm) was obtained from the treatment 1.5 mg/L NAA+1.5 mg/L BAP with the variety Granola and the lowest length (1.80 cm) was obtained from 1.5 mg/L NAA+0.5 mg/L BAP with the same variety (Table 11).

Table 9: Effect of different varieties on length of shoots/plantlet and number of leaves/plantlet at different days after inoculation

Variety	Length of shoots/plantlet (cm)				Number of leaves/plantlet			
	7 DAI	14 DAI	21 DAI	28 DAI	7 DAI	14 DAI	21 DAI	28 DAI
Granola	0.99	2.38	3.88	6.76	0.00	0.77	3.08	8.32
Diamant	0.96	1.80	3.15	6.51	0.00	0.00	1.71	7.51
Asterix	1.03	1.76	2.73	5.68	0.00	0.47	1.64	5.08
SE±	0.081	0.124	0.136	0.202	0.00	0.09	0.189	0.344
Max	1.03	2.38	3.88	6.76	0.00	0.77	3.08	8.32
Min	0.96	1.76	2.73	5.68	0.00	0.09	1.64	5.08
LSD	0.110	0.186	0.267	0.445	0.027	0.000	0.242	0.431
Level of significance	NS	**	**	**	**	**	**	**

Table 10: Effect of different hormones on length of shoots/plantlet and number of leaves/plantlet at different days after inoculation

Hormone	Length of shoots/plantlet (cm)				Number of leaves/plantlet			
	7 DAI	14 DAI	21 DAI	28 DAI	7 DAI	14 DAI	21 DAI	28 DAI
T1=Normal MS	1.13	3.38	5.31	7.63	0.00	0.00	1.47	8.93
T2=0.5BAP	2.06	3.81	4.59	6.23	0.00	0.00	0.00	6.27
T3=1.0BAP	2.49	4.09	5.33	8.83	0.00	0.00	5.33	11.87
T4=1.5BAP	2.59	4.11	4.83	6.78	0.00	1.67	6.27	11.33
T5=2.0BAP	2.54	3.90	4.72	6.23	0.00	0.67	3.67	6.00
T6= 1.0NAA	0.00	0.17	2.09	4.89	0.00	0.00	0.73	4.80
T7=1.0NAA+0.5BAP	0.31	0.68	1.87	5.47	0.00	0.53	1.27	5.80
T8=1.0NAA+1.0BAP	0.00	0.25	2.09	3.65	0.00	0.00	0.00	2.93
T9=1.0NAA+1.5BAP	0.00	0.15	1.15	2.24	0.00	0.00	0.00	0.00
T10=1.0NAA+2.0BAP	0.31	0.66	1.26	2.14	0.00	0.00	0.00	0.00
T11= 1.5NAA	0.00	1.02	3.79	8.33	0.00	0.00	1.80	8.47
T12=1.5NAA+0.5BAP	0.61	2.57	4.41	7.54	0.00	0.00	1.87	7.67
T13=1.5NAA+1.0BAP	0.00	0.40	1.57	7.08	0.00	0.00	1.60	7.67
T14=1.5NAA+1.5BAP	2.88	4.23	5.83	10.13	0.00	3.33	6.33	13.27
T15=1.5NAA+2.0BAP	0.00	0.28	2.75	8.71	0.00	0.00	1.80	8.53
SE±	0.081	0.124	0.136	0.202	0.00	0.086	0.189	0.344
Max	2.88	4.23	5.83	10.13	0.00	3.33	6.33	13.27
Min	0.31	0.15	1.15	2.14	0.00	0.53	0.73	2.93
LSD	0.245	0.415	0.598	0.994	0.060	0.000	0.541	0.964
Level of significance	**	**	**	**	**	**	**	**

Table 11: Combined effect of different varieties and different hormones on length of shoots/plantlet and number of leaves/plantlet at different days after inoculation

Variety	Hormone	Length of shoots/plantlet (cm)				Number of leaves/plantlet			
		7 DAI	14 DAI	21 DAI	28 DAI	7 DAI	14 DAI	21 DAI	28 DAI
Granola	T1=Normal MS	2.22	6.10	7.20	9.30	0.00	0.00	1.80	7.60
	T2=0.5BAP	1.92	4.38	5.28	8.30	0.00	0.00	0.00	9.00
	T3=1.0BAP	2.10	3.58	5.20	9.80	0.00	0.00	8.00	15.40
	T4=1.5BAP	2.72	5.10	6.14	7.34	0.00	1.00	6.00	11.00
	T5=2.0BAP	2.68	4.20	4.78	6.74	0.00	2.00	11.00	13.00
	T6= 1.0NAA	0.34	2.02	4.52	10.20	0.00	0.00	1.20	6.20
	T7=1.0NAA+0.5BAP	0.66	1.40	2.66	4.16	0.00	1.60	1.80	6.60
	T8=1.0NAA+1.0BAP	0.00	0.42	2.70	4.36	0.00	0.00	0.00	3.40
	T9=1.0NAA+1.5BAP	0.00	0.46	1.84	2.70	0.00	0.00	0.00	0.00
	T10=1.0NAA+2.0BAP	0.14	0.78	1.74	2.38	0.00	0.00	0.00	0.00
	T11= 1.5NAA	0.00	1.16	2.82	8.38	0.00	0.00	2.40	8.80
	T12=1.5NAA+0.5BAP	0.00	0.00	0.88	1.80	0.00	0.00	1.80	7.60
	T13=1.5NAA+1.0BAP	0.00	1.20	1.76	7.50	0.00	0.00	2.00	8.00
	T14=1.5NAA+1.5BAP	2.08	4.12	7.60	10.72	0.00	7.00	8.20	20.00
	T15=1.5NAA+2.0BAP	0.00	0.84	3.12	7.40	0.00	0.00	2.00	7.20
Diamant	T1=Normal MS	2.80	3.26	4.64	6.80	0.00	0.00	2.60	15.40
	T2=0.5BAP	1.88	3.42	4.18	5.00	0.00	0.00	0.00	8.80
	T3=1.0BAP	2.10	4.10	5.77	10.00	0.00	0.00	5.00	11.60
	T4=1.5BAP	2.88	3.68	4.26	7.32	0.00	0.00	3.00	7.00
	T5=2.0BAP	3.18	4.22	5.22	6.28	0.00	0.00	0.00	5.00
	T6= 1.0NAA	0.00	2.88	5.62	8.60	0.00	0.00	1.00	8.20
	T7=1.0NAA+0.5BAP	0.26	0.64	2.48	9.14	0.00	0.00	1.00	5.80
	T8=1.0NAA+1.0BAP	0.00	0.34	2.28	3.62	0.00	0.00	0.00	3.00
	T9=1.0NAA+1.5BAP	1.30	3.00	5.50	9.00	0.00	0.00	0.00	0.00
	T10=1.0NAA+2.0BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T11= 1.5NAA	0.00	0.00	1.62	4.02	0.00	0.00	2.00	9.80
	T12=1.5NAA+0.5BAP	0.00	0.00	2.40	5.36	0.00	0.00	1.80	7.20
	T13=1.5NAA+1.0BAP	0.00	0.00	1.60	6.70	0.00	0.00	1.60	7.80
	T14=1.5NAA+1.5BAP	0.00	1.46	6.86	9.80	0.00	0.00	5.60	13.00
	T15=1.5NAA+2.0BAP	0.00	0.00	3.34	9.70	0.00	0.00	2.00	10.00
Asterix	T1=Normal MS	2.60	3.34	4.08	6.80	0.00	0.00	0.00	3.80
	T2=0.5BAP	2.38	3.62	4.30	5.38	0.00	0.00	0.00	1.00
	T3=1.0BAP	3.26	4.58	5.20	6.70	0.00	0.00	3.00	3.00
	T4=1.5BAP	2.18	3.56	4.08	5.68	0.00	4.00	10.00	16.00
	T5=2.0BAP	2.78	3.28	4.16	5.68	0.00	0.00	0.00	0.00
	T6= 1.0NAA	1.50	2.82	3.08	3.82	0.00	0.00	0.00	0.00
	T7=1.0NAA+0.5BAP	0.00	0.00	0.46	3.12	0.00	0.00	1.00	5.00
	T8=1.0NAA+1.0BAP	0.00	0.00	1.28	2.96	0.00	0.00	0.00	2.40
	T9=1.0NAA+1.5BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T10=1.0NAA+2.0BAP	0.80	1.20	2.04	4.04	0.00	0.00	0.00	0.00
	T11= 1.5NAA	0.00	0.44	1.68	6.80	0.00	0.00	1.00	6.80
	T12=1.5NAA+0.5BAP	0.00	0.50	3.00	7.50	0.00	0.00	2.00	8.20
	T13=1.5NAA+1.0BAP	0.00	0.00	1.36	7.04	0.00	0.00	1.20	7.20
	T14=1.5NAA+1.5BAP	0.00	3.02	4.38	10.68	0.00	3.00	5.00	14.40
	T15=1.5NAA+2.0BAP	0.00	0.00	1.80	9.02	0.00	0.00	1.40	8.40
	SE±	0.081	0.124	0.136	0.202	0.000	0.086	0.189	0.344
	Max	3.26	6.10	7.60	10.72	0.00	7.00	11.00	20.00
	Min	0.14	0.34	0.88	1.80	0.00	1.00	1.00	3.00
	LSD	0.425	0.719	1.036	1.722	0.104	0.000	0.937	1.670
	Level of significance	**	**	**	**	**	**	**	**

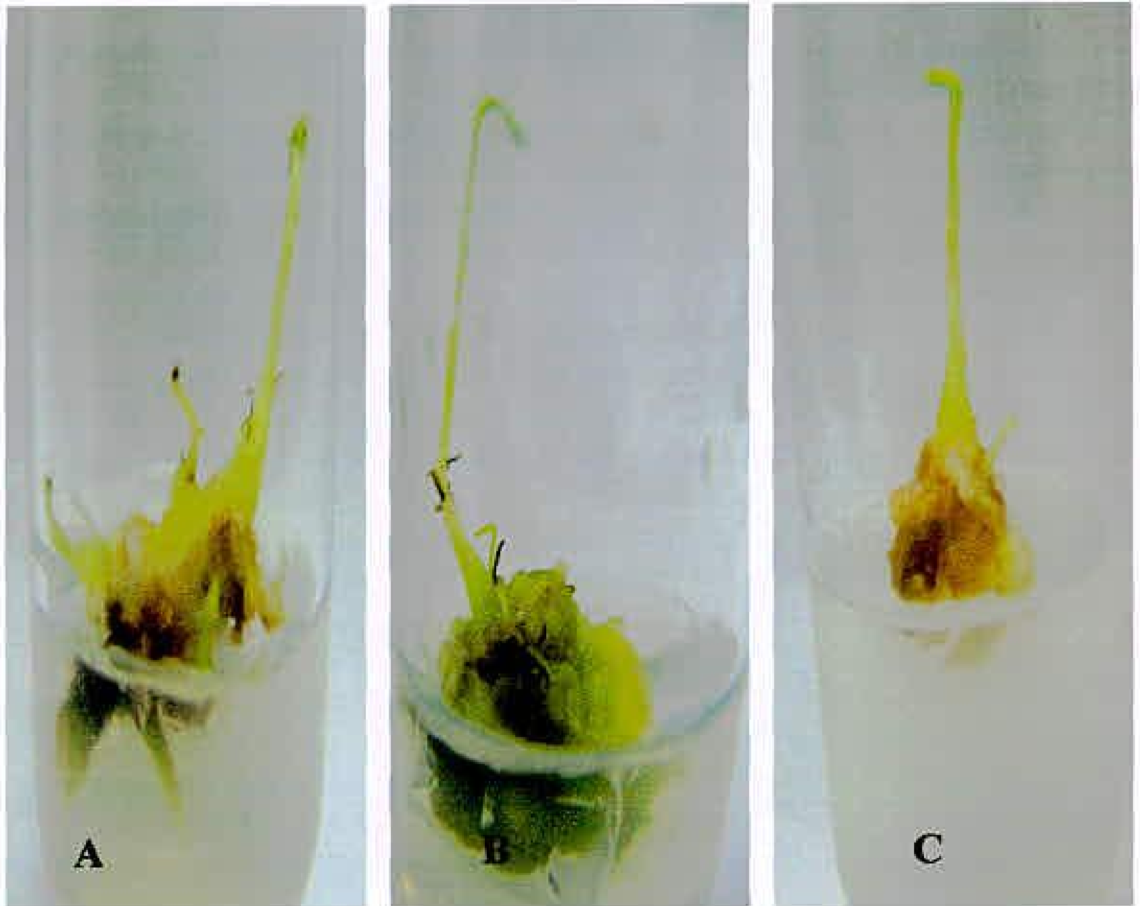


Plate 13. Maximum length of shoot at 7 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

Plate 14. Maximum length of shoot at 14 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

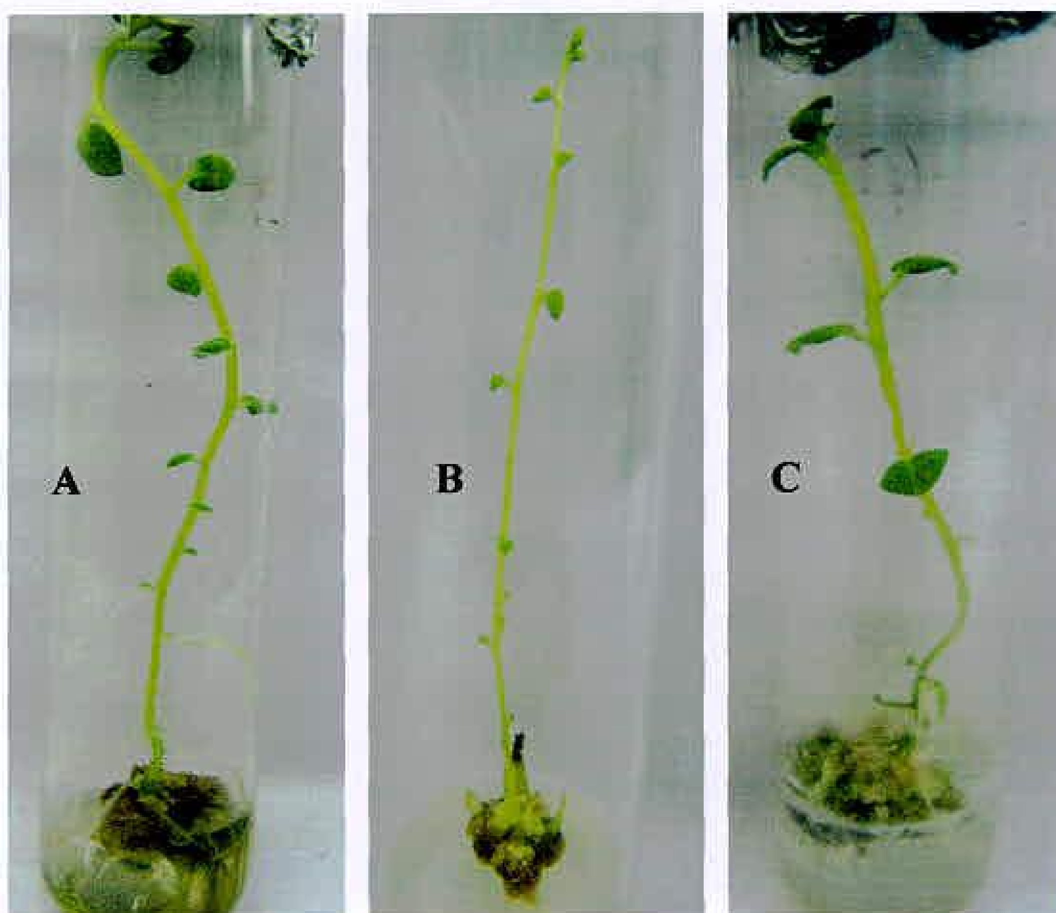


Plate 15. Maximum length of shoot at 21 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

Plate 16. Maximum length of shoot at 28 DAI on MS media supplemented with 1.5 mg/L NAA+ 1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

4.2.9. Number of leaves/plantlet

The number of leaves/plantlet was recorded after 7, 14, 21 and 28 days of cultured on MS media containing different concentrations of NAA and BAP. The results have been presented in Table 9-11 and Plate 17-20.

The significant effect of three potato varieties was found in respect of number leaves/plantlets. At 7 DAI no leaf was formed in any variety. At 14, 21 and 28 DAI the maximum number of leaves/plantlet was showed in variety Granola (0.77, 3.08 and 8.25 leaves/plantlet, respectively) and the minimum number of leaves/plantlet was found in variety Asterix (0.47, 1.64 and 5.08 leaves/plantlet at 14, 21 and 28, DAI, respectively) (Table 9).

Different concentrations of NAA and BAP on number of leave also statistically different days. The highest number of leaves/plantlet at 14, 21 and 28 days (3.33, 6.33 and 13.27 leaves/plantlet, respectively) was produced with the concentration of 1.5 mg/L NAA+1.5 mg/L BAP. MS media combined with 1.0 mg/L NAA+1.0 mg/L BAP produced the lowest number of leaves/plantlet (2.93 leaves/plantlet 28 DAI) (Table 10).

The combined effect of varieties and different concentrations of NAA and BAP on number of leaves/plantlet was statistically significant at 14, 21 and 28 days. At 7 DAI no leaf is formed in any variety. At 28 DAI the highest number of leaves/plantlet were recorded in variety Granola (20.00 leaves/plantlet) at the concentration of 1.5 NAA mg/L+1.5 mg/L BAP. The lowest number of leaves/plantlet was showed in variety Asterix (1.00 leaves/plantletlet) with ms media combined with 0.5 mg/L BAP (Table 11).

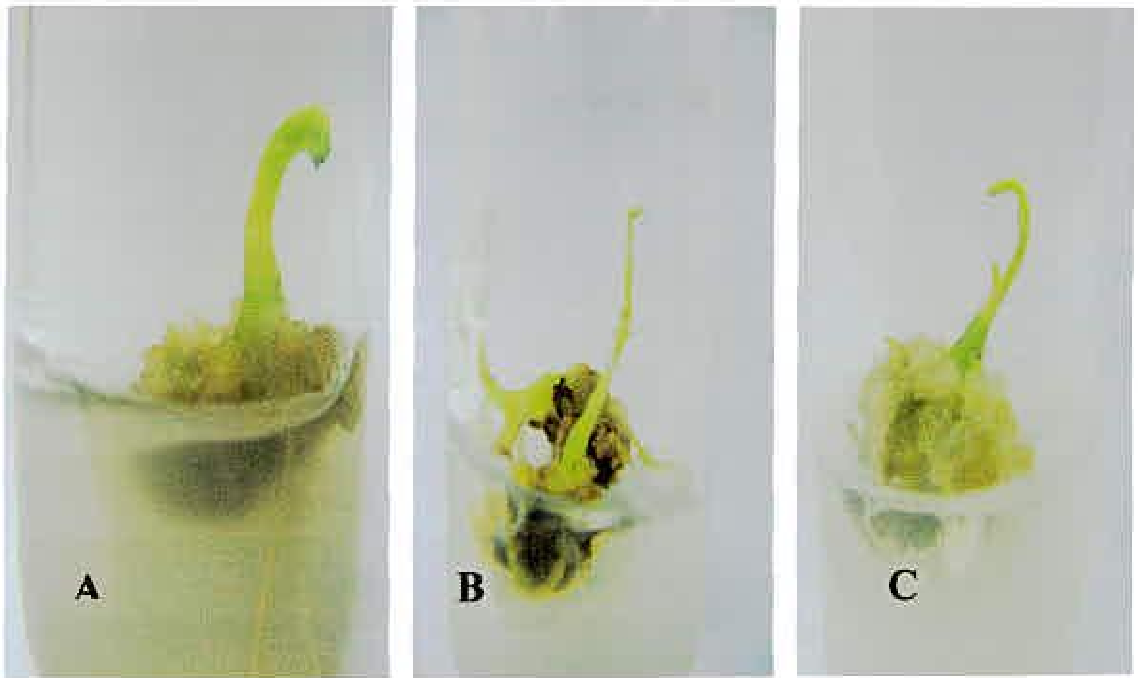


Plate 17. No leaf initiated at 7 DAI on MS media supplemented with 1.5 mg/L NAA+1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

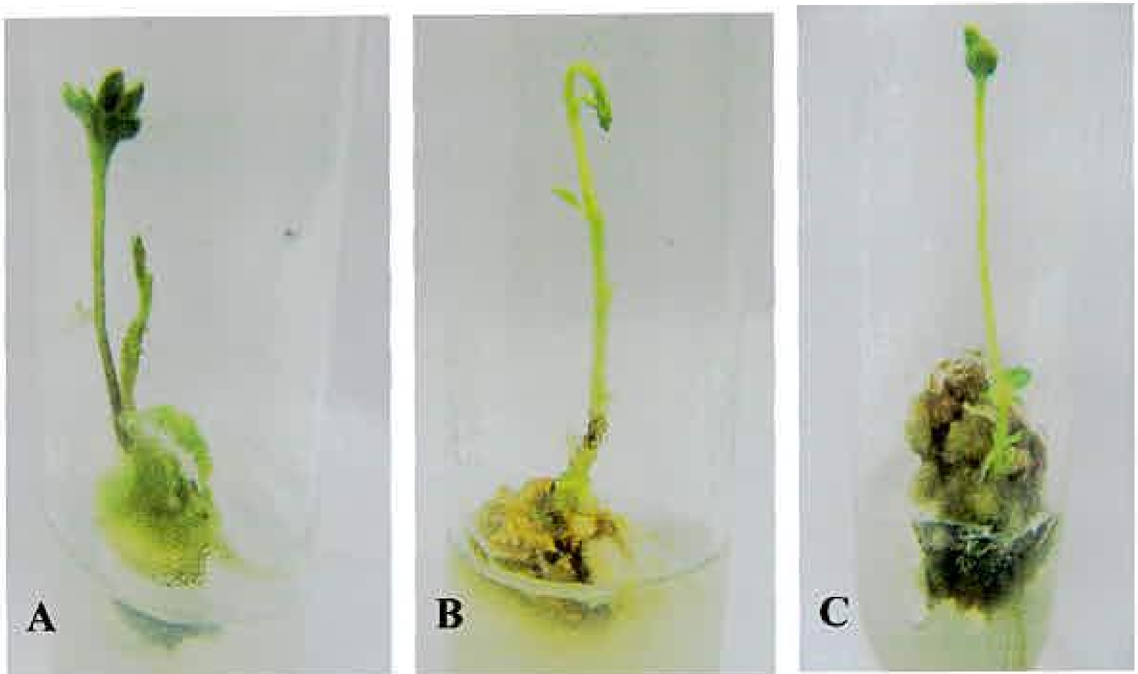


Plate 18. Maximum number of leaf initiated at 14 DAI on MS media supplemented with 1.5 mg/L NAA+1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

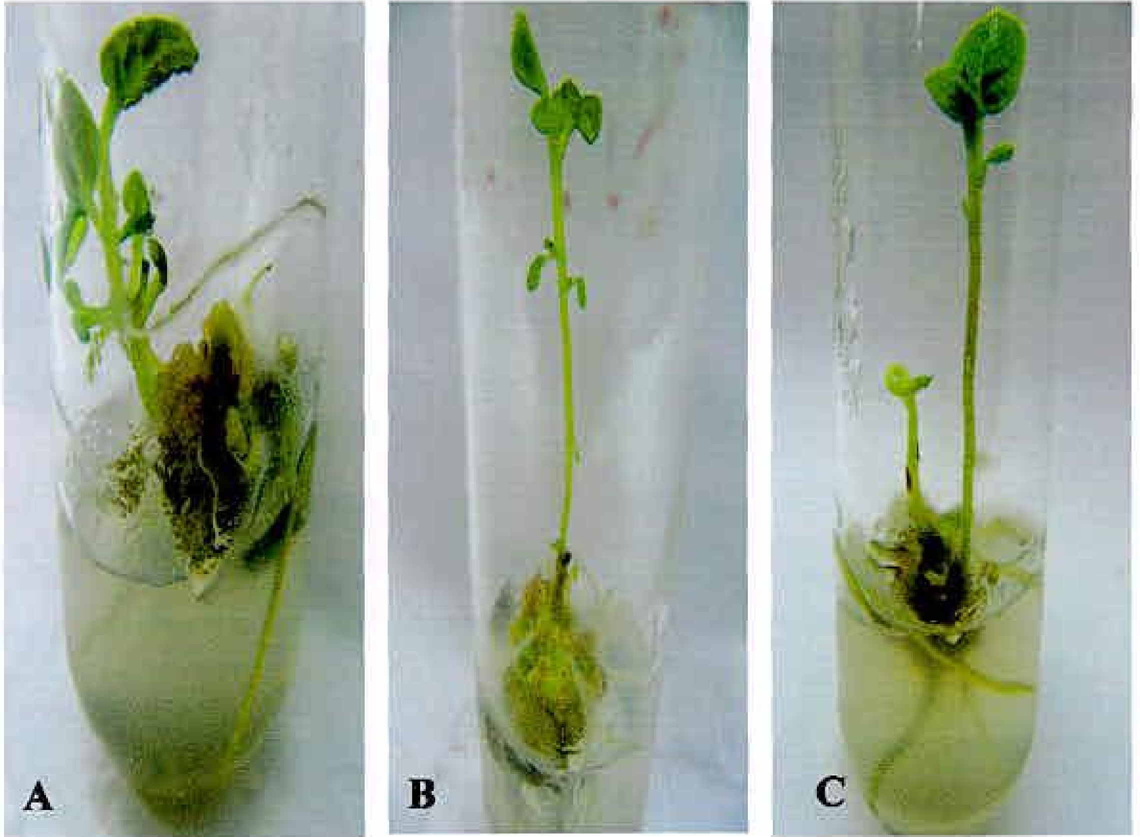


Plate 19. Maximum number of leaf initiated at 21 DAI on MS media supplemented with 1.5 mg/L NAA+1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

Plate 20. Maximum number of leaf initiated at 28 DAI on MS media supplemented with 1.5 mg/L NAA+1.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

4.2.10. Days to root initiation

Days to root initiation was recorded after 7, 14, 21 and 28 days of cultured on MS media containing different concentrations of NAA and BAP. The results have been presented in Table 12-14 and Appendix IX.

The results of major effect of varieties on days to root initiation have been presented in Table 12. The days to root initiaiton varied significantly among the three varieties. The maximum days was recorded in variety Diamant (15.48 days) whereas the minimum days was noticed in variety Asterix (12.73 days).

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

Variety	Days to root initiation	Number of roots/plantlet			
		7 DAI	14 DAI	21 DAI	28 DAI
Granola	15.01	0.92	1.31	2.41	6.43
Diamant	15.48	1.37	1.61	3.19	6.97
Asterix	12.73	0.43	0.49	1.41	5.68
SE±	0.828	0.107	0.132	0.240	0.350
Max	15.48	1.37	1.61	3.19	6.97
Min	12.73	0.43	0.49	1.41	5.68
LSD	0.407	0.120	0.157	0.309	0.438
Level of significance	**	**	**	**	**

There was significant influence of different hormone combination and concentrations of NAA and BAP on the days to root initiation (Table 13 and Plate 21). The minimum days (0.93 days) was observed on MS media with 1.5 mg/L NAA+0.5 mg/L BAP. The maximum days (29.27 days) was observed for root initiation on MS media without hormone.

Table 13: Effect of different hormones on days to root initiation and number of roots/plantlet at different days after inoculation

Hormone	Days to root initiation	Number of roots/plantlet			
		7 DAI	14 DAI	21 DAI	28 DAI
T1=Normal MS	29.27	0.00	0.00	0.00	4.20
T2=0.5BAP	-	0.00	0.00	0.00	0.00
T3=1.0BAP	24.67	0.00	0.00	0.00	1.87
T4=1.5BAP	23.00	0.00	0.00	1.53	5.00
T5=2.0BAP	19.07	0.00	0.00	1.13	1.40
T6= 1.0NAA	9.33	0.67	1.00	3.53	7.40
T7=1.0NAA+0.5BAP	19.40	0.47	0.53	2.73	7.40
T8=1.0NAA+1.0BAP	3.40	2.80	3.13	3.93	7.00
T9=1.0NAA+1.5BAP	1.80	2.67	3.13	4.20	5.27
T10=1.0NAA+2.0BAP	2.73	3.13	3.47	3.73	4.93
T11= 1.5NAA	3.47	0.67	3.62	5.60	10.40
T12=1.5NAA+0.5BAP	0.93	3.20	5.13	7.53	14.53
T13=1.5NAA+1.0BAP	25.33	0.00	0.00	0.00	7.80
T14=1.5NAA+1.5BAP	25.80	0.00	0.00	1.07	9.40
T15=1.5NAA+2.0BAP	27.93	0.00	0.00	0.07	6.80
SE±	0.828	0.107	0.132	0.240	0.350
Max	29.27	3.20	5.13	7.53	14.53
Min	0.93	0.47	0.53	1.07	1.40
LSD	0.911	0.268	0.352	0.691	0.980
Level of significance	**	**	**	**	**

The potato varieties and different levels of hormones showed significant interaction in relation to the days to root initiation. The maximum days (30.40 days) to root initiation on MS media containing no hormone in cv. Diamant. The minimum days was observed in the variety Asterix (1.20 days) on MS media containing 1.5 mg/L NAA+0.5 mg/L BAP (Table 14).

Table 14: Combined effect of different varieties and different hormones on days to root initiaiton and number of roots/plantlet at different days after inoculation

Variety	Hormone	Days to root initiaiton	Number of roots/plantlet			
			7 DAI	14 DAI	21 DAI	28 DAI
Granola	T1=Normal MS	28.60	0.00	0.00	0.00	2.00
	T2=0.5BAP	-	0.00	0.00	0.00	0.00
	T3=1.0BAP	29.80	0.00	0.00	0.00	4.60
	T4=1.5BAP	28.80	0.00	0.00	0.00	5.60
	T5=2.0BAP	28.80	0.00	0.00	3.40	4.20
	T6= 1.0NAA	2.20	1.40	1.60	4.00	14.20
	T7=1.0NAA+0.5BAP	27.20	0.00	0.00	1.80	7.00
	T8=1.0NAA+1.0BAP	21.00	0.00	0.00	2.20	8.60
	T9=1.0NAA+1.5BAP	2.20	2.80	4.20	6.20	8.60
	T10=1.0NAA+2.0BAP	2.20	1.20	2.00	2.00	2.60
	T11= 1.5NAA	2.00	4.00	4.40	5.00	6.00
	T12=1.5NAA+0.5BAP	6.60	2.00	2.00	6.40	10.60
	T13=1.5NAA+1.0BAP	22.80	0.00	0.00	0.00	2.20
	T14=1.5NAA+1.5BAP	27.80	0.00	0.00	0.00	3.40
	T15=1.5NAA+2.0AP	28.60	0.00	0.00	0.00	3.00
Diamant	T1=Normal MS	30.40	0.00	0.00	0.00	5.0
	T2=0.5BAP	-	0.00	0.00	0.00	0.00
	T3=1.0BAP	28.40	0.00	0.00	0.00	1.00
	T4=1.5BAP	28.20	0.00	0.00	0.00	5.00
	T5=2.0BAP	-	0.00	0.00	0.00	0.00
	T6= 1.0NAA	6.40	2.00	3.00	6.60	8.00
	T7=1.0NAA+0.5BAP	25.40	0.00	0.00	0.80	4.00
	T8=1.0NAA+1.0BAP	26.60	0.00	0.00	0.40	8.60
	T9=1.0NAA+1.5BAP	1.60	6.20	6.40	7.20	9.20
	T10=1.0NAA+2.0BAP	1.60	4.20	4.20	4.60	5.20
	T11= 1.5NAA	7.60	5.80	7.60	12.60	15.60
	T12=1.5NAA+0.5BAP	2.80	2.40	5.40	16.80	19.40
	T13=1.5NAA+1.0BAP	26.80	0.00	0.00	0.00	9.80
	T14=1.5NAA+1.5BAP	4.40	2.40	3.00	3.80	8.40
	T15=1.5NAA+2.0BAP	25.20	0.00	0.00	0.20	3.80
Asterix	T1=Normal MS	0.00	0.00	0.00	0.00	0.00
	T2=0.5BAP	0.00	0.00	0.00	0.00	0.00
	T3=1.0BAP	-	0.00	0.00	0.00	0.00
	T4=1.5BAP	21.40	0.00	0.00	3.00	4.00
	T5=2.0BAP	-	0.00	0.00	0.00	0.00
	T6= 1.0NAA	1.60	1.00	1.00	1.80	2.00
	T7=1.0NAA+0.5BAP	21.40	0.00	0.00	5.60	11.20
	T8=1.0NAA+1.0BAP	21.40	0.00	0.00	2.00	3.80
	T9=1.0NAA+1.5BAP	2.60	1.40	2.40	3.60	4.20
	T10=1.0NAA+2.0BAP	4.40	2.00	2.00	2.00	3.00
	T11= 1.5NAA	1.60	1.40	2.40	3.60	14.60
	T12=1.5NAA+0.5BAP	1.20	5.80	7.60	12.60	16.40
	T13=1.5NAA+1.0BAP	26.40	0.00	0.00	0.00	9.40
	T14=1.5NAA+1.5BAP	3.80	2.00	2.00	3.20	12.40
	T15=1.5NAA+2.0BAP	30.00	0.00	0.00	0.00	7.60
	SE±	0.828	0.107	0.132	0.240	0.350
	Max	30.40	6.20	7.60	16.80	19.40
	Min	1.20	1.00	1.00	0.20	1.00
	LSD	1.578	0.464	0.609	1.197	1.698
	Level of significance	**	**	**	**	**

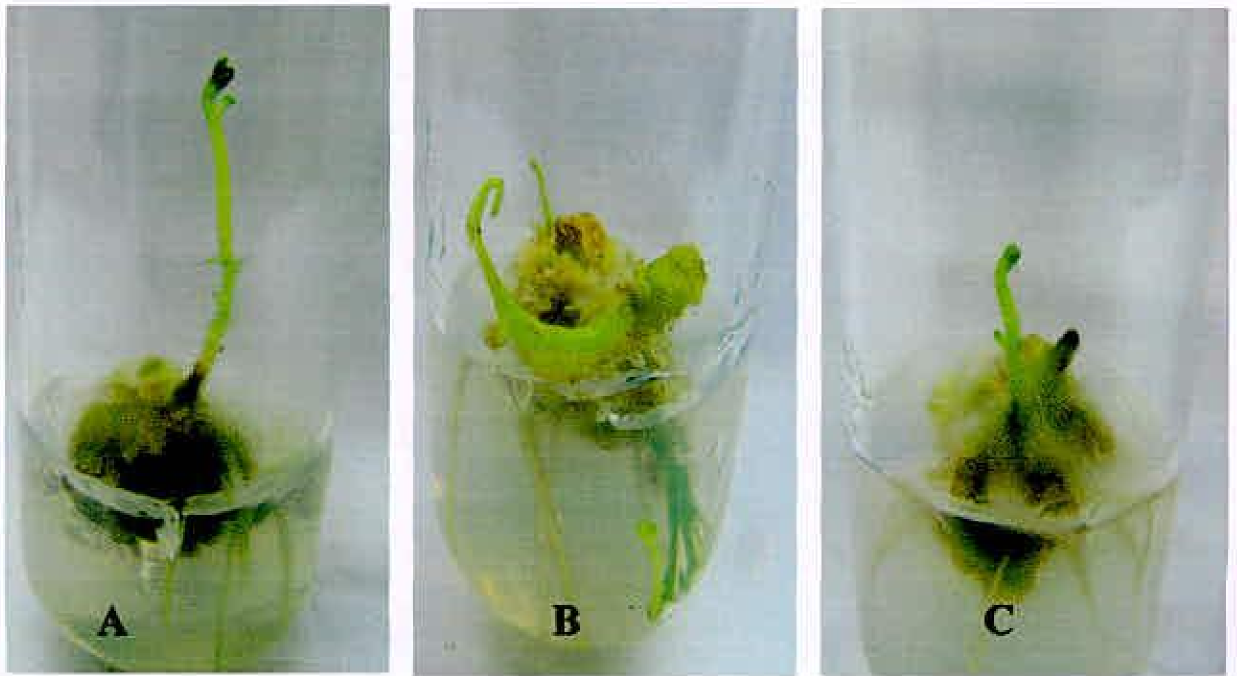


Plate 21. Initiated root at 7 DAI on MS media supplement with 1.5 mg/L NAA+0.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

4.2.11. Number of roots/plantlet

The number of roots/plantlet was recorded after 7, 14, 21 and 28 days of culture. A significant variation was found on number of roots due to different potato varieties (Appendix IX). At 7, 14, 21 and 28 days of culture, the variety Diamant produced maximum number of roots/plantlet (1.37, 1.61, 3.19 and 6.97 roots/plantlet) and variety Asterix produced minimum number of roots (0.43, 0.49. 1.41 and 5.68 roots/plantlet at 7, 14, 21 and 28 days, respectively) (Table 12).With the progress of time, the number of roots/plantlet increased gradually in all varieties.

The main effect of different concentrations of NAA and BAP on number of roots/plantlet was statistically significant. The highest number of root (3.20, 5.13, 7.53 and 14.53 roots/plantlet at 7, 14, 21 and 28 days, respectively) was observed in treatment 1.5 mg/L NAA+0.5 mg/L BAP. Whereas the minimum number of root (0.47 and 0.53 roots/ plantlet at 7 and 14 days, respectively) was observed in treatment 1.0 mg/L NAA+0.5 mg/L BAP. And in 28 days minimum (1.40 roots/plantlet) was observed in the treatment 2.0 mg/L BAP (Table 13 and Plate 22-24).

The combined effect of variety and different concentrations of NAA and BAP on number of roots/plantlet showed statistically significant result at 7, 14, 21 and 28 DAI (Table 14). At 21 and 28 days of culture, the highest number of roots/plantlet was noted in the variety Diamant (16.80 and 19.40 roots/plantlet, respectively) at the concentration of 1.5 mg/L NAA+0.5 mg/L BAP and at 28 DAI the lowest number of roots/plantlet (1 root/plantlet) was also recorded in the variety Diamant on 1.0 mg/L BAP. Auxin is essential for root initiation in culture.

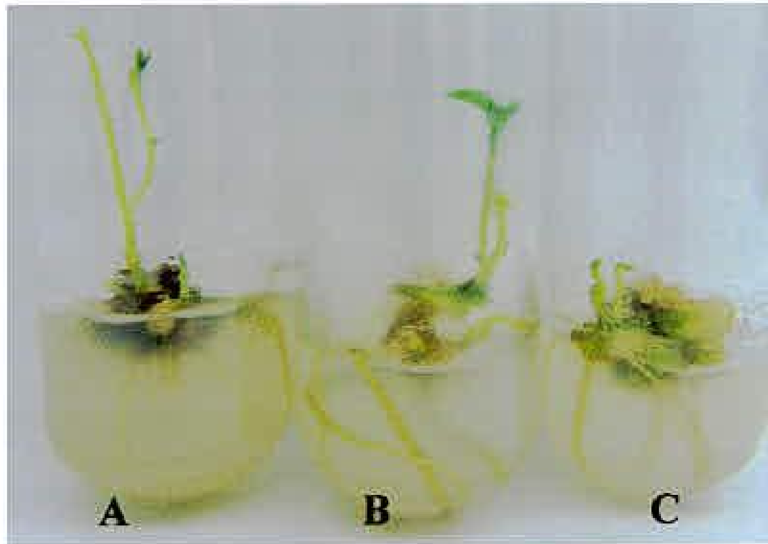


Plate 22. Maximum number of root with longest root initiated at 14 DAI on MS media supplemented with 1.5 mg/L NAA+0.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

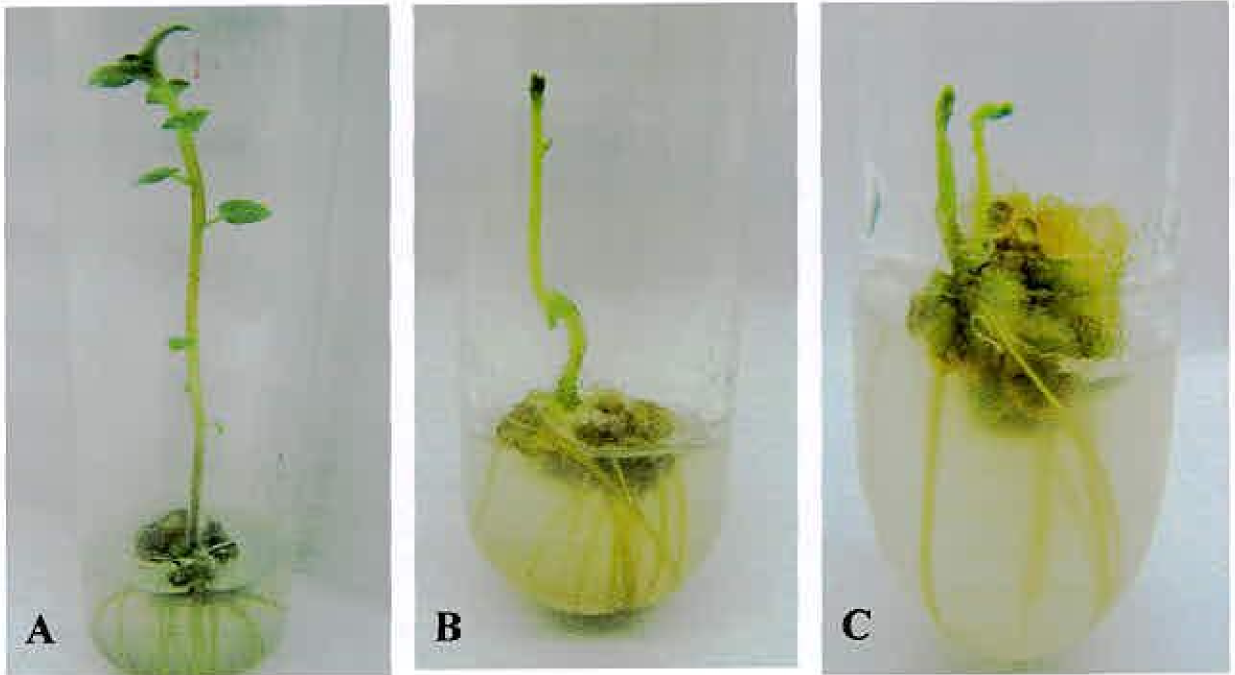


Plate 23. Maximum number of root with longest root initiated at 21 DAI on MS media supplemented with 1.5 mg/L NAA+0.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

Plate 24. Maximum number of root with longest root initiated at 28 DAI on MS media supplemented with 1.5 mg/L NAA+0.5 mg/L BAP in A. Granola, B. Diamant and C. Asterix

4.2.12. Length of roots/plantlet

The length of roots per plantlet was recorded after 7, 14, 21 and 28 days of cultured on MS media containing different concentrations of NAA and BAP. The results have been presented in Appendix X, Table 15-17 and Plate 22-24.

The influence of these potato varieties was significance in respect of length of root at 7, 14, 21 and 28 days of culture. The maximum length of root at 7, 14, 21 and 28 days of culture was recorder in variety Granola (0.78, 1.01, 1.59 and 4.60 cm, respectively) and the minimum length of root was found in the variety Asterix (0.57, 0.70, 1.16 and 2.34 cm at 7, 14, 21 and 28 DAI, respectively) (Table 15).

Length of root was recorded at 7, 14, 21 and 28 days of culture, also differed significantly due to the main effect of different concentrations of NAA and BAP. The maximum length of roots (3.41, 4.73, 5.18 and 7.45 cm at 7, 14, 21 and 28 DAI respectively) was recorded at the concentration of 1.5 mg/L NAA+0.5 mg/L BAP at 28 days of culture whereas the minimum length of root (0.67 cm) was produced on MS media containing 1.0 mg/L BAP (Table 16). Sanavy *et al.* (2003) reported that low concentrations of auxin positively influenced root length.

The results of the present experiment showed that there was significant effect on root length due to the combined effect of varieties and different concentrations of NAA and BAP at 7, 14, 21, and 28 days of culture (Table 17). The maximum length of root (5.04, 5.40 and 14.42 cm at 14, 21, and 28 DAI) was recorded in the variety Granola at the concentration of 1.5 mg/L NAA+0.5 mg/L BAP. The minimum length of root (1.36 cm at 28 days of culture) was found in the same variety on MS media containing 1.0 mg/L BAP.

Table 15: Effect of different varieties on length of roots/plantlet at different days after inoculation

Variety	Length of roots/plantlet (cm)			
	7 DAI	14 DAI	21 DAI	28 DAI
Granola	0.78	1.01	1.59	4.60
Diamant	0.70	0.94	1.26	2.99
Asterix	0.57	0.70	1.16	2.34
SE±	0.071	0.095	0.109	0.198
Max	0.78	1.01	1.59	4.60
Min	0.57	0.70	1.16	2.34
LSD	0.020	0.014	0.162	0.080
Level of significance	**	**	**	**

Table 16: Effect of different hormones on length of roots/plantlet at different days after inoculation

Hormone	Length of roots/plantlet (cm)			
	7 DAI	14 DAI	21 DAI	28 DAI
T1=Normal MS	0.00	0.00	0.00	2.14
T2=0.5BAP	0.00	0.00	0.00	0.00
T3=1.0BAP	0.00	0.00	0.00	0.95
T4=1.5BAP	0.00	0.00	0.96	2.23
T5=2.0BAP	0.00	0.00	0.67	0.67
T6= 1.0NAA	0.67	0.67	1.76	5.06
T7=1.0NAA+0.5BAP	0.40	0.52	1.25	3.59
T8=1.0NAA+1.0BAP	0.56	0.67	1.77	4.34
T9=1.0NAA+1.5BAP	1.97	2.57	3.08	7.03
T10=1.0NAA+2.0BAP	1.77	2.56	2.79	3.43
T11= 1.5NAA	1.49	1.58	1.77	3.75
T12=1.5NAA+0.5BAP	3.41	4.73	5.18	7.45
T13=1.5NAA+1.0BAP	0.00	0.00	0.00	3.67
T14=1.5NAA+1.5BAP	0.00	0.00	1.13	2.43
T15=1.5NAA+2.0BAP	0.00	0.00	0.21	2.97
SE±	0.071	0.095	0.109	0.198
Max	3.41	4.73	5.18	7.45
Min	0.40	0.52	0.21	0.67
LSD	0.046	0.032	0.362	0.178
Level of significance	**	**	**	**

Table 17: Combined effect of different varieties and different hormones on length of roots/plantlet at different days after inoculation

Variety	Hormone	Length of roots/plantlet (cm)			
		7 DAI	14 DAI	21 DAI	28 DAI
Granola	T1=Normal MS	0.00	0.00	0.00	2.06
	T2=0.5BAP	0.00	0.00	0.00	0.00
	T3=1.0BAP	0.00	0.00	0.00	1.36
	T4=1.5BAP	0.00	0.00	0.00	3.00
	T5=2.0BAP	0.00	0.00	2.00	2.00
	T6= 1.0NAA	1.62	1.72	1.92	2.12
	T7=1.0NAA+0.5BAP	0.00	0.00	0.30	3.16
	T8=1.0NAA+1.0BAP	0.00	0.00	1.30	2.16
	T9=1.0NAA+1.5BAP	2.58	3.12	3.68	10.00
	T10=1.0NAA+2.0BAP	1.50	1.74	2.34	4.00
	T11= 1.5NAA	1.20	1.56	2.04	4.32
	T12=1.5NAA+0.5BAP	3.12	5.04	5.40	14.42
	T13=1.5NAA+1.0BAP	0.00	0.00	0.00	3.86
	T14=1.5NAA+1.5BAP	0.00	0.00	0.00	3.02
	T15=1.5NAA+2.0BAP	0.00	0.00	0.00	2.74
Diamant	T1=Normal MS	0.00	0.00	0.00	2.28
	T2=0.5BAP	0.00	0.00	0.00	0.00
	T3=1.0BAP	0.00	0.00	0.00	1.50
	T4=1.5BAP	0.00	0.00	0.00	2.08
	T5=2.0BAP	0.00	0.00	0.00	0.00
	T6= 1.0NAA	2.00	2.00	2.00	2.46
	T7=1.0NAA+0.5BAP	0.00	0.00	1.74	5.12
	T8=1.0NAA+1.0BAP	0.00	0.00	0.46	2.50
	T9=1.0NAA+1.5BAP	1.80	2.88	3.86	4.34
	T10=1.0NAA+2.0BAP	1.60	3.02	3.04	3.16
	T11= 1.5NAA	1.37	1.47	3.65	7.36
	T12=1.5NAA+0.5BAP	1.68	2.00	2.00	2.40
	T13=1.5NAA+1.0BAP	0.00	0.00	0.00	3.44
	T14=1.5NAA+1.5BAP	1.52	1.64	1.96	3.80
	T15=1.5NAA+2.0BAP	0.00	0.00	0.64	3.86
Asterix	T1=Normal MS	0.00	0.00	0.00	0.00
	T2=0.5BAP	0.00	0.00	0.00	0.00
	T3=1.0BAP	0.00	0.00	0.00	0.00
	T4=1.5BAP	0.00	0.00	1.44	2.18
	T5=2.0BAP	0.00	0.00	0.00	0.00
	T6= 1.0NAA	1.52	1.70	1.70	2.34
	T7=1.0NAA+0.5BAP	0.00	0.00	1.70	2.48
	T8=1.0NAA+1.0BAP	0.00	0.00	1.62	2.64
	T9=1.0NAA+1.5BAP	3.52	4.54	5.00	5.00
	T10=1.0NAA+2.0BAP	2.20	2.92	3.00	3.12
	T11= 1.5NAA	*1.43*	*1.79*	*2.67*	*3.94*
	T12=1.5NAA+0.5BAP	3.58	4.60	5.14	12.72
	T13=1.5NAA+1.0BAP	0.00	0.00	0.00	2.70
	T14=1.5NAA+1.5BAP	1.32	1.38	2.88	6.08
	T14=1.5NAA+2.0BAP	0.00	0.00	0.00	2.30
	SE±	0.071	0.095	0.109	0.198
	Max	3.58	5.04	5.40	14.42
	Min	1.20	1.38	0.30	1.36
	LSD	0.079	0.056	0.628	0.308
	Level of significance	**	**	**	**

4.3. Experiment 3: Acclimatization and establishment of plantlets in soil

4.3.1. Multiplication of plantlet for acclimatization

From the overall experiment, it was revealed that three treatments were best in respect with rapid callus induction (1.0 mg/L NAA +1.5 mg/L BAP), maximum shoot/plantlet (1.5 mg/l NAA+1.5 mg/L BAP) and rapid root (1.5 mg/L NAA+0.5 mg/L) initiation. Plants from these treatments were subcultured on normoal MS media for further multiplication (Table 18 and Plate 25-26).

Table 18: Subcultured plant for multiplication on normal MS media

Variety	Source treatment	No. of shoots/ plantlet	Length of shoots/ plantlet (cm)	No. of leaves/ plantlet	No. of roots/ plantlet	Length of roots/ plantlet (cm)
Granola	1.0NAA+1.5BAP	3.8	8.2	9.2	6.6	9.76
	1.5NAA+1.5BAP	1.6	7.8	9.4	7.2	6
	1.5NAA+0.5BAP	3.4	7.8	8.8	6.8	8.86
Daimant	1.0NAA+1.5BAP	1	8.46	7.8	7.4	6.96
	1.5NAA+1.5BAP	1	8.6	9.8	7.6	4.06
	1.5NAA+0.5BAP	1	8	9	7.2	6
Asterix	1.0NAA+1.5BAP	1.4	7.5	7	3.8	3.66
	1.5NAA+1.5BAP	1.4	7	7	4.4	3.9
	1.5NAA+0.5BAP	1.2	8.28	6.8	4	3.76

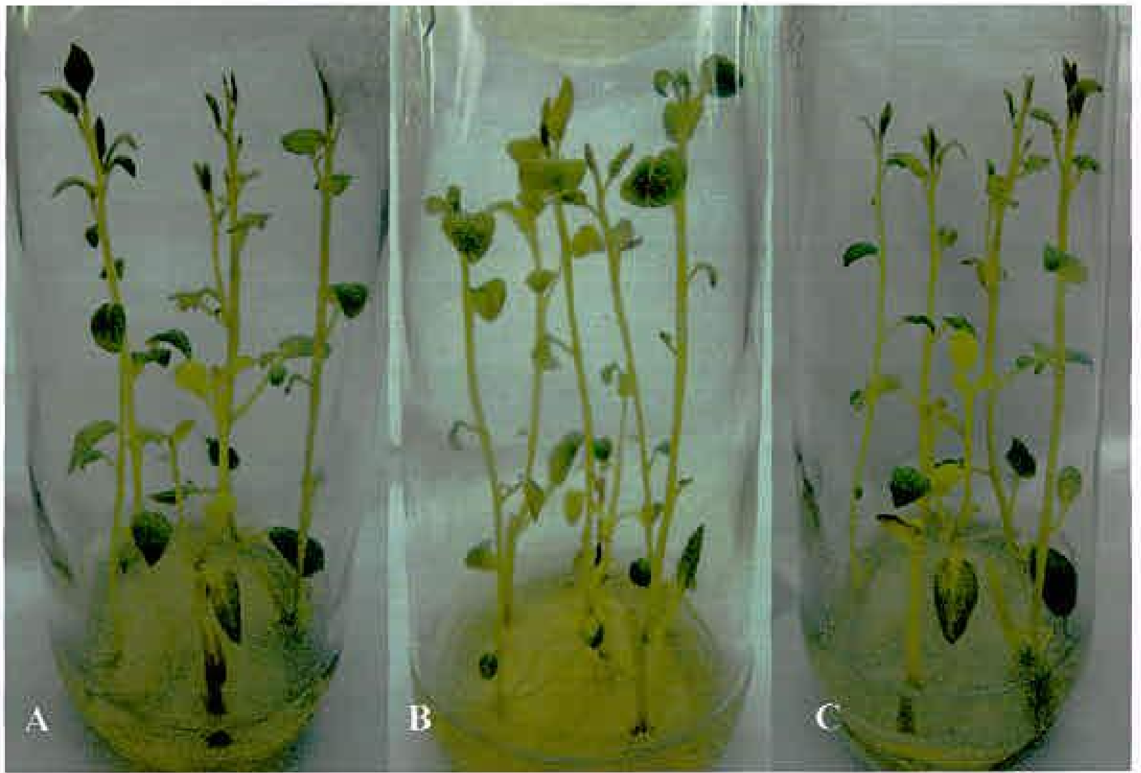


Plate 25. Regenerated subcultured plantlets of A. Granola, B. Diamant and C. Asterix

Plate 26. Multiplication of the variety Granola

4.3.2. Preparation of pot and transplantation

Potting mixture containing garden soil, sand and cowdung in the ratio 1:2:1 was mixed properly and autoclaved one hour in 121°C for 20 minutes at 1.16 kg/cm^2. After cooling, the soil mixture was taken into 10 cm pots for growing the pots *in vivo* condition. When the plantlets became5-8 cm in height with sufficient shoot and root system, they were taken out from the vials without damaging any roots. Medium attached to the roots was gently washed out running tap water to prevent further micorbial infection. The plantlets were then transplanted to pot containing potting mixture mention above. Immediately after transplantation the plants along with the pots were covered with moist polythene bag to prevent desiccation. To reduce sudden shock the pots were kept in a growth room for 7-15 days under controlled environments (Plate 27). The interior of the polythene bags was sprayed with distilled water at every 24 hours to maintain high humidity around the plantlets. After 2-3 days, the polythene bags were gradually perforated to expose the plants to natural environment. The polythene bags were completely removed after 10-15 days when the plantlets, appeared to be self- sustainable. At this stage, the plantlets were placed in natural environment for 3-10 hours daily (Plate 28-29). The highest survival rate 82.22% found in cv. Granola (Plate 30) and the lowest survival rate. 66.67% % found in cv. Asterix (Table 19).

Table 19: Survival rate of *in vitro* regenerated plantlets of three potato varieties

Acclimatization	Variety	No. of transplanted plants	No. of plants survives	Survival rate (%)
Initially small hole of plastic tray at growth chamber	Granola	45	37	82.22%
	Daimant	45	33	73.33%
	Asterix	45	30	66.67%
Subsequently when moved to soil in open atmosphere	Granola	37	35	94.60%
	Daimant	33	31	93.94%
	Asterix	30	27	90.00%

Finally, after 15-20 days, they were transferred to the net house for hardening and after proper hardening the plantlets were transplanted to the soil (Plate 31). As soon as new leaves started to initiate, plants were watered with ordinary tap water. Gradually the plantlets were adapted to the soil. In the open atmosphere plantlets of cv. Granola gave the highest survival rate 94.60% and the lowest was 90.00% in cv. Asterix (Table 18).

Plate 27 . *In vivo* acclimatization of regenerated plantlets in growth chamber

Plate 28. Plantlets were placed in natural environment for hardening

Plate 29. *In vivo* establishment of regenerated plantlets in open atmosphere

Plate 30. Variety Granola showed the highest survival rate

Plate 31. A. Hardening of plantlets in net house; B. Transplantation of healthy plantlets to the soil

Plate 31. A. Hardening of plantlets in net house; B. Transplantation of healthy plantlets to the soil

CHAPTER 5
SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUSION

The present experiment was conducted in the Genetics and Plant Breeding Laboratory and the Tissue culture laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, during the period of January 2011 to December 2011 from sprout of three potato varieties namely Granola, Diamant, Asterix to establish *in vitro* callus induction and plant regeneration using different concentration and combination of NAA and BAP. The experiment was conducted at Completely Randomized Design (CRD) with 5 replications. The concentrations of NAA were 1.0 and 1.5 mg/L, and for BAP were 0.5, 1.0, 1.5 and 2.0 mg/L. The combine effect of both the hormones were also studied. To investigate the effect of different treatments of this experiment, the parameters were recorded on, callus color and texture, days to callus induction, percentage of callus induction, size of callus, fresh weight of callus, days to shoot initiation, number of shoots/plantlet, number of leaves/plantlet, length of shoots/plantlet, days to root initiation, number of roots/plantlet and length of roots/plantlet.

It revealed that days to callus induction, size and weight of callus were significantly influenced by different varieties and different concentrations of NAA and BAP. The maximum (5.94 days) and minimum (4.46 days) days to callus induction were observed in the varieties Asterix and Diamant, respectively. Treatment 1.0 mg/L NAA showed the maximum (7.93 days) days to callus induction while it was the minimum (3.53 days) in 1.0 mg/L NAA+1.5 mg/L BAP. In the interaction effect of varieties and hormones, variety Asterix with 1.5 mg/L NAA took the maximum (14.00 days) days to callus induction whereas it was the minimum (3.00 days) in variety Granola with 1.0 mg/L NAA+1.5 mg/L BAP.

The highest size (1.61 cm) having maximum weight (1.91 gm) of callus and the lowest size (1.42 cm) having minimum weight (1.30 gm) of callus were found in the variety Granola and Asterix, respectively. Treatment 1.5 mg/L NAA showed the largest callus size (1.94 cm) with maximum weight (2.08 gm). The smallest (1.14 cm)

and the minimum weight (1.15 gm) of callus was observed in 1.0 mg/L NAA+1.5 mg/L BAP. At 28 DAI, variety Diamant showed the largest callus size (2.10 cm) with the maximum weight (2.54 gm) in 1.5 mg/L NAA and in 1.0 mg/L NAA+0.5 mg/L BAP in variety Granola found the smallest callus size (0.95 cm) with the minimum weight (0.64 gm) of callus.

The different concentrations of hormone significantly influenced the days to shoot initiation and days to root initiation with number of shoot and roots/plantlet. The treatment combination 1.5 mg/L NAA+1.5 mg/L BAP required minimum days (6.87 days) to shoot initiation in respect with the highest (4.87 shoots/plantlet) and the longest (10.13 cm) length of shoots/plantlet whereas the treatment combination 1.5 NAA mg/L+0.5 mg/L required minimum days (0.93 days) to root initiation with the highest (14.53 roots/plantlet) and longest (7.45 cm) roots/plantlet.

When varieties were taken under consideration, influence on all the parameters under study was found significant. Variety Granola took the minimum days (13.17 days) to shoot initiation. The highest number of shoot (3.00 shoots/plantlet) having the longest shoot (6.76cm) and the maximum number of leaf (8.32 leaves/plantlet) was also noticed in the same variety. This variety also produced the longest (4.60 cm) length of root at 28 DAI.

Combined effect of varieties and different concentrations of hormones showed significant variations on days to shoot and root initiation, number and length of shoots and roots. The minimum days to shoot (5.40 days) and root (1.20 days) initiation were in treatment combination 1.5 mg/L NAA+1.5 mg/L BAP in variety Granola and 1.5 mg/L NAA+0.5 mg/L BAP in variety Asterix, respectively. The maximum number of shoot (7.00 shoots/plantlet) with the longest shoot (10.72cm) was observed in treatment combination 1.5 mg/L NAA+1.5 mg/L BAP with variety Granola. Beside this, the maximum (19.40 roots/plant) and the longest (14.42 cm) root was found in the hormonal treatment combination 1.5 mg/L NAA+0.5 mg/L BAP in variety Diamant and Granola, respectively.

The overall experimental findings revealed that the variety Granola was the most responsive to GA_3 application for sprouting and days to sprout initiation gradually decreased with the increased concentration of GA_3. The results of the present investigation also indicated that potato cultivars Granola, Diamant and Asterix could be successfully micropropagated using 1.0 mg/L NAA +1.5 mg/L BAP for rapid callus induction and 1.5 mg/L NAA for getting large sized callus. Considering the findings of the present study, MS media supplemented with 1.5 mg/L NAA+1.5 mg/L BAP for rapid shoot regeneration and 1.5 mg/L NAA+0.5 mg/L was found to be the best for rapid root formation. It was also revealed that auxin (NAA) alone and auxin (NAA) with cytokinin (BAP) in combination could induce callus but BAP alone could not produce callus.

For acclimatization, plantlets were transplanted from culture media to soil in tray with small hole in the growth chamber, where percentage of survival was the highest (82.22%) in cv. Granola and minimum percentage of survival (66.67%) was in Asterix. After transplanting the plantlets from small hole of tray to soil, 94.60% of *in vitro* regenerated plantlets in cv. Granola and 90.00% in cv. Asterix survived in the extreme garden condition.

The protocol developed from the present study may be useful for large scale production of healthy and disease free planting materials of potato commercially. This protocol could be used for *in vitro* breeding programme. Also the findings of the study may be used for genetic transformation for the improvement of potato using biotechnological approach.

CHAPTER 6
REFFERENCS

CHAPTER 6

REFERENCES

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CHAPTER 7
APPENDICES

APPENDICES

Appendix I. Composition and stock solution of MS (Murashige and Skoog 1962) medium

Stock-1	Major salts (10*)	mg/L	g/L
	KNO_3	1900	19.00
	NH_4NO_3	1650	16.50
	$MgSO_4.7H_2O$	370	3.370
	$CaCl_2.2H_2O$	440	4.40
	KH_2PO_4	170	1.70
Stock-2	**Minor salts (100*)**		
	KI	0.83	83
	H_3BO_3	6.2	620
	$MnSO_4.4H_2O$	22.3	2230
	$ZnSO_4.7H_2O$	8.6	860
	$Na_2MoO_4.2H_2O$	0.25	25
	$CuSO_4.5H_2O$	0.025	2.5
	$CoCl_2.6H_2O$	0.025	2.5
Stock-3	**Iron EDTA soln. (100*)**		
	$FeSO_4.7H_2O$	27.8	2.78
	$Na_2EDTA.2H_2O$	37.3	3.73
Stock-4	**Organics (100*)**		
	Myo-inositol	100	10000
	Nicotinic acid	0.5	50
	Pyridoxin HCl	0.5	50
	Thiamine HCl	0.1	10
	Glycin	2.0	200

Appendix II. Analysis of variance of days to callus induction and breadth of callus

Sources of variation	Degrees of freedom	Days to callus induction	Breadth of callus (cm)			
			7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	13.927**	2.889^{NS}	0.269^{NS}	0.065^{NS}	0.742*
Factor B	9	6.525**	2.883**	0.238*	0.732**	1.734**
AB	18	7.601**	1.826**	0.249**	0.327**	0.259^{NS}
Error	120	1.077	0.042	0.114	0.133	0.187

**=Significant at 1% level
*=Significant at 5% level
^{NS}=Non significant

Appendix III. Analysis of variance of length of callus

Sources of variation	Degrees of freedom	Length of callus (cm)			
		7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	0.002^{NS}	0.025^{NS}	0.042^{NS}	0.742*
Factor B	9	0.02**	0.146**	0.741**	1.734**
AB	18	0.014**	0.089**	0.204*	0.259^{NS}
Error	120	0.006	0.029	0.117	0.187

**=Significant at 1% level
*=Significant at 5% level
^{NS}=Non significant

Appendix IV. Analysis of variance of size of callus

Sources of variation	Degrees of freedom	Size of callus (cm)			
		7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	0.011^{NS}	0.094^{NS}	0.053^{NS}	0.567**
Factor B	9	0.033**	0.162**	0.653**	1.035**
AB	18	0.03**	0.136**	0.218**	0.16^{NS}
Error	120	0.007	0.049	0.077	0.118

**=Significant at 1% level
*=Significant at 5% level
^{NS}=Non significant

Appendix V. Analysis of variance of fresh weight of callus

Sources of variation	Degrees of freedom	Fresh weight of callus (gm)	
		Initial weight (14 DAI)	Final weight (30 DAI)
Factor A	2	0.937**	4.5**
Factor B	9	0.436**	1.477**
AB	18	0.32**	0.784**
Error	120	0.081	0.316

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

Appendix VI. Analysis of variance of days to shoot initiation and number of shoots/plantlet

Sources of variation	Degrees of freedom	Days to shoot initiation	Number of shoots/plantlet			
			7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	75.538**	3.36**	11.338**	20.013**	25.191**
Factor B	14	684.532**	7.019**	14.452**	14.792**	21.961**
AB	28	169.4**	1.308**	1.433**	2.423**	4.672**
Error	180	9.544	0.158	0.376	0.453	1.289

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

Appendix VII. Analysis of variance of length of shoots/plantlet

Sources of variation	Degrees of freedom	Length of shoots/plantlet (cm)			
		7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	0.102^{NS}	9.195**	29.177**	28.415**
Factor B	14	20.246**	45.802**	41.603**	84.17**
AB	28	1.004**	1.786**	6.011**	16.973**
Error	180	0.116	0.332	0.689	1.903

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

Appendix VIII. Analysis of variance of number of leaves/plantlet

Sources of variation	Degrees of freedom	Number of leaves/plantlet			
		7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	0.000**	11.373**	49.551**	213.098**
Factor B	14	0.000**	12.916**	74.199**	242.932**
AB	28	0.000**	5.921**	19.713**	65.326**
Error	180	0.000	0.007	0.564	1.791

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

Appendix IX. Analysis of variance of days to root initiation and number of roots/plantlet

Sources of variation	Degrees of freedom	Days to root initiation	Number of roots/plantlet			
			7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	162.004**	16.813**	25.124**	59.284**	31.613**
Factor B	14	1960.284**	25.484**	43.195**	83.481**	245.236**
AB	28	232.6**	5.78**	6.534**	51.48**	84.218**
Error	180	1.598	0.138	0.238	0.92	1.851

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

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Appendix X. Analysis of variance of length of roots/plantlet

Sources of variation	Degrees of freedom	Length of roots/plantlet (cm)			
		7 DAI	14 DAI	21 DAI	28 DAI
Factor A	2	0.837**	1.976**	4.64**	101.13**
Factor B	14	15.765**	29.324**	30.815**	66.828**
AB	28	1.113**	1.526**	4.258**	29.492**
Error	180	0.004	0.002	0.253	0.061

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