IN VITRO PLANT REGENERATION OF THREE VARIETIES OF POTATO (*Solanum tuberosum* L.) FROM EPICOTYL

THESIS

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MST. KAZUMI IFFAT

Student No. : 0905034
Registration No. : 0905034
Session : 2009-10
Semester : Winter

MASTER OF SCIENCE (MS)
IN
BIOTECHNOLOGY

DEPARTMENT OF GENETICS AND PLANT BREEDING
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR

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DEDICATED

TO
MY

BELOVED PARENTS

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The author

IN VITRO PLANT REGENERATION OF THREE VARIETIES OF POTATO (*Solanum tuberosum* L.) FROM EPICOTYL

ABSTRACT

The experiment was carried out during the period from December 2009 to April 2010, in the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur. Callus induction, shoot formation, root induction and plantlet regeneration of three standard potato varieties viz. Cardinal, Diamant and Patrones from epicotyl of potato were carried out in this experiment. For callus induction, four levels of IAA (0.0, 0.1, 0.5 and 1.0 mg/L) and BAP (0.0, 1.0, 3.0 and 7.0 mg/L) and NAA (0.0, 0.01 and 0.1 mg/L) were used. Callus induction from epicotyl of potato was positively influenced by 0.5-1.0 mg/L IAA + 3.0-7.0 mg/L BAP, producing 100% calli within 6-12 days of culture. This treatment produced 27.72 - 53.50g callus with maximum growth rate of 133.75 mg/day. Plantlet regeneration markedly enhanced by BAP. All the three varieties showed better performance on plantlet regeneration with 1.0-2.0 mg/L BAP alone or 1.0 mg/L BAP + 0.01-0.1 mg/L NAA, producing maximum shoot per explant (2.01) and plant height (4.78 cm).

ABBREVIATIONS

The following abbreviations have been used throughout the whole thesis

2, 4-D	: 2, 4-dichlorophenoxy acetic acid
BA	: Benzyl adenine
BAP	: 6 – Benzyl Amino Purine
BBS	: Bangladesh Bureau of Statistics
cm	: Centimeter
CV	: Coefficient of variance
DAC	: Days after culture
DAI	: Days after incubation
et al.	: With other
FAO	: Food and Agriculture Organization
Fig	: Figure
gm	: Gram
IAA	: Indole Acetic Acid
IBA	: Indole Butyric Acid
Kn	: Kinetin
LSD	: Least Signification Difference Test
mg/L	: Miligram per Litre
ml	: Mililitre
MS	: Murashige and Skoog
NAA	: α-napthalene acetic acid
pH	: Negative logarithm of hydrogen ion (H+) concentration
Var.	: Variety
viz.	: Namely
wt.	: Weight

CONTENTS

CONTENTS (CONT'D)

CONTENTS (CONT'D)

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

The potato is a starchy, annual herbaceous dicotyledonous, tuberous crop from the perennial *Solanum tuberosum* of the Solanaceae family (also known as the nightshades) which contains approximately 160 tuber bearing species. The word potato may refer to the plant itself as well as the edible tuber. In this region of the Andes mountain of South America where it has served as a main item in the diet of native people of millennia. Despite being first introduced outside the Andes region four centuries ago, today potatoes have become an integral part of much of the world's cuisine and are the world's fourth-largest food crop, following rice, wheat, and maize.

The potato (*Solanum tuberosum* L.) is a vegetable crop of major economic importance worldwide (Beukema, 1990). It is the fourth most cultivated food crop after wheat, rice and maize and therefore, the most important dicotyledonous and tuber crop (Jones, 1994). As such potato growers produce about 315 million metric tons of potato annually. Of them People's Republic of China (70 million metric ton), Russia (39 million metric ton), India (24 million metric ton), United States (20 million metric ton), Ukraine (19 million metric ton), Germany (10 million metric ton), Poland (9 million metric ton), Belgium (8 million metric ton), Netherlands (7 million metric ton) and France (6 million metric ton) remain in the top listed countries of potato production (FAO, 2006).

Potato provides energy 321 kJ (77 kcal), carbohydrates 19 g, starch 15 g, dietary fiber 2.2 g, fat 0.1 g, protein 2 g, water 75 g, thiamine (Vit. B_1) 0.08 mg (6%), riboflavin (Vit. B_2) 0.03 mg (2%), niacin (Vit. B_3) 1.1 mg (7%), vitamin B_6 0.25 mg (19%), vitamin C 20 mg (33%), Calcium 12 mg (1%), Iron1.8 mg (14%),

Magnesium 23 mg (6%), Phosphorus 57 mg (8%), Potassium 421 mg (9%), Sodium 6 mg (0%) and some other trace elements in per 100g.

The area and production of potato in Bangladesh have increased to a greater extent over the last decades, but the yield per unit area remained more or less static. In Bangladesh 6648 thousand metric tons of potatoes are produced from 401 thousand hectares of land in 2007 – 2008 (BBS, 2009).The average yield of potato in Bangladesh is 16.57 thousand metric ton/ha which is much lower than that of many growing countries of the world.

The crop is usually propagated as sexually by means of tubers that are the underground stems of the plant. However, with the conventional method of vegetative propagation, potatoes are often prone to pathogen such as fungi, bacteria, and viruses, thereby resulting in poor quality and yields. Consequently much attention has been focused on the *in vitro* production of virus free potatoes (Mellor and Stace-Smith, 1977; Diurdjina *et al.*, 1997).

Obtaining quantities of clean planting material has been a major barrier to potato production in many developing countries. Seed tuber multiplication is slow and disease tends to increase with each multiplication. Plant tissue culture is now used to produce disease free good quality seed tubers. A system of seed production utilizing tissue culture and rapid multiplication techniques has been proved successful in South China, Vietnam and Taiwan. The same is likely to be equally successful in Bangladesh (Zaag, 1990).The regeneration of plants from cell and tissue culture is an important and essential component of biotechnology that is required for the genetic manipulation of plants. High frequency regeneration of plants from *in vitro* cultured tissues and cells is a prerequisite for successful application of tissue culture and genetic engineering technologies or crop improvement. Many attempts have been made to enhance the frequency of plant

regeneration from potato callus and a lot of research has been devoted to investigate the factors affecting plant regeneration. Great progress has been made in potatoes for plant regeneration in recent years (Ehsanpour and Jones, 2000; Figert *et al.*, 2000; Ahn *et al.*, 2001).

For many years, tissue culture has been applied to improve potato production by means of micropropagation, pathogen elimination and germplasm conservation (Slack, 1988). Micropropagation allows rapid multiplication of potato clones in a short duration under disease free controlled environment and on a year round basis (Ahloorvalia, 1994). On the other hand, micropropagated plants, when cultured under suitable conditions, produce *in vitro* microtubers.

Therefore, the *in vitro* technology combined with traditional practices have enhanced the commercial production of virus free seeds an important pre-requisite to maximize yield in potato (Faccioli and Colombarini, 1996). It is also the first major food crop where biotechnology has been successfully applied for virus elimination (Bajaj and Sopory, 1986). Hussain and Rashid (1991) obtained an average of about 60.5% of virus free plants in Cardinal with the highest number 80% after heat treatment.

Based on the above information, the present experiment was undertaken to study the *in vitro* regeneration potential of potato, with the following objectives -

i) To observe callusing ability in some standard potato cultivars
ii) To regenerate plantlets from calli of potato
iii) To study the genotypic variation for induction of callus and plantlets

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

The potato (*Solanum tuberosum* L.) as a vegetatively propagated crop is prone to cumulative infection by bacteria, fungi, viruses and viriods a process commonly known as degeneration. Virus diseases have been recognized as a limiting factor in potato production worldwide. The successful production of potatoes for nutritional and seed purposes demands the control of these viruses which can not be sufficiently controlled by any physical or chemical agent. Tissue culture is an important technique of biotechnology and has a potential to improve the quality and quantity of vegetatively propagated potato plants. Developing countries of the world are now applying biotechnological approach in this field is not so fast. Therefore, the literatures, which are most relevant and available to the present study, have been reviewed under the following heads.

2.1 Concept of tissue culture

Conventional techniques of plant improvement are lengthy process. As a result plant tissue culture and biotechnological techniques are gradually being accepted as the most efficient techniques for crop improvement. Tissue culture has been developed as new and powerful tool for the improvement of different crops (Carlson, 1975) and received wide attention of modern scientist. When explants of a plant are grown in a defined medium, an undifferentiated collection of cells arise, which is called callus. The medium can be subsequently manipulated to obtain root, shoot and embryos. Direct regeneration from explants without callus is also responded thus the process is known as regeneration. Plant regeneration from in vitro cultures is a perquisite of many plant genetic transformation techniques.

2.2 Tissue culture of *Solanum tuberosum* L.

Although tissue culture of *Solanum tuberosum* L. was initiated at sixty decades but marked development and refinement and also the potential uses of tissue culture technique in the improvement of potato have been made during the last two decades (Ahn *et al.*, 2001; Yildirim *et al.*, 2002; Schafer Menuhr *et al.*, 2003; Omidi and Shahpiri, 2003; Roy, 2004).

2.2.1 Callus induction

Smolenskaya (1989) reported production of primary callus by in vitro culture of potato leaf and stem explants on medium with various supplements. Explant of the primary callus was cultured on the same medium but without benzylaminopurine (BAP) and NAA or both of these growth regulators.

Warch *et al.* (1989) mentioned the initiation of primary callus from leaf explants of the North American cultivar Viking and Norgold-M formation stolon like structures while those of Red lasoda formed both roots and stolon like structures. After three transfer 30% of Red Lasoda callus eventually regenerated 1-3 shoots per axillary bud in Red Lasoda, Norgold-M and Viking respectively.

Annenkov and Beluga (1991) reported the rate of callus accumulation depend on variety, type of explant, nutrient medium, light, temperature and season. A high rate of callus formation was observed in three varieties and much less in other five also best results were obtained with 20h illumination. Rapid callus cell growth observed in autumn while in spring the callus growth tended to give way to development of organs.

Garica and Martinez (1995) evaluated when somatic embryos were obtained from stem nodal section of in vitro cultured potato plants 4-8 mm long. Calli were

initiated in MS salts supplemented with 0.5 mg pyridoxin, 2 mg glycine, 0.5 mg yeast extract, 4 mg 2, 4-D, 25 g sucrose and 2 g gelrite/L and pH was 5.5.

Fomenko *et al.* (1998) conducted an experiment where the callus formation in tuber, stem and leaf explants of potato hybrid 78563-76 and cultivars Rosinka and Otrada on MS medium supplemented with various growth regulators and they reported that different explants and different genotypes reacted differently to the growth regulators in the medium in terms of callus formation.

More *et al.* (2001) investigated the biological activity of brassinosteriod analogues MH5 and Hiobras-6 (BB-6) to determine their effects on embryogenic callus formation of potato cv. Desiree. The appropriate doses (0.0001-0.25 mg a. i. /L) of MH5 as BB-6 and possible substitutes or hormonal complements were also established. Two control media were used 3 mg 2, 4-D and 0.84 mg kinetin and 0.5 mg 2, 4-D and 2 mg Kinetin/L. The best callus formation was observed in medium II supplemented with 0.1 mg MH5 + 0.01 mg BB-6/L. There was no callus formation when MH5 and BB-6 were used as substitutes for 2, 4-D. Callus formation was observed when MH5 and BB-6 were used as kinetin substitutes.

Schafer *et al.* (2003) initiated callus cultures of potato cv. Clarissa from the end pieces of internodes explants producing somatic embryos then cultured on solid MS media supplemented with 1 mg 2, 4-D/L. They reported the cell lines did not develop somatic embryos when cultured in hormone free medium. Somatic embryos formed only in cultures that were inoculated with a low number of cells. A high concentration of zeatin (2.5 mg/L) and a low level of polyphenols were significant. In vitro plants were regenerated from somatic embryos after transferring in a hormone free medium. The yield of somatic embryos significantly increased when cells were encapsulated in hollow beads. Embryo formation was

not only observed on solid medium but also in shaken cultures in which they further developed into plantlets.

2.2.1 Effects of genotype variability

Tick and Kollist (1992) revealed callus induction potentiality of leaf, stem and tuber explants of 11 varieties on 7 modifications of MS medium, and on maintenance of the callus on 12 modifications of the medium with supplements. All the varieties showed callus forming ability in the optimum medium, but there were differences in the rate of stability of callus formation with Adreta, Suliev, Olive and prevalent showing high callus growth rate in all applications.

An experiment was conducted by Miklovicova *et al.* (1999) where apical and axilary meristems of 7 dihaploid potato genotypes cultured on medium V were studied for microproilagation and tuberisation. Genotype VL 173/82 was the most productive. Medium supplemented with 1% saccharose had a positive influence on regeneration while on medium containing 100 g coumarin/L on microtuber initiation.

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 genotypes and medium. Second order interactions were proved statistically significant. The best undifferentiated growth of friable calli on leaf explants was observed after 4 weeks on medium containing 0.25 mg kinetin and 5.0 mg 2, 4-D/L.

The effects of cultivar and explant on callus induction in leaf and internode explants of six potato cultivars (Agria, Cosmos Sante, Concord, Ajax and Diamant) were observed by Omidi and Shahpiri (2003). Leaf and internode

explants planted on MS medium, supplemented with 5 mg/L 2, 4-D and 0.25 mg/L kinetin. The effect of cultivar explant and their interaction on frequency of callus induction was insignificant, while the effects of these factors on the initiation of time of callus induction were significant.

2.2.1.2 Effect of explants

Goyal *et al.* (1990) found multiple shoot formation in cotyledon explants was found to be most efficient on MS medium with high kinetin/benzyladenine and low IAA concentration. Hypocotyl and redicle explants had relatively less shoot morphogenetic potential.

Bhalla and Smith (1998a) conducted an experiment and observed root and hypocotyl explants produced more callus than cotyledon explants and also more responsive to shoot regeneratioans, high percentage >71% and >80% for hypocotyl and roots respectively of shoot initiation from these explants. In addition, root and hypocotyl explants also produced more shoots per explants than cotyledon explants.

2.2.1.3 Effect of growth regulators

Montanqlli *et al.* (1989) studied the regeneration pattern of potato using a range of culture media for growing potato tuber explants. Results from cv. Desiree and Spunta showed that 100% of Desiree and 90% of Spunta explants formed callus of which 14 and l2.5% respectively regenerated on medium containing 2.5% sugar, 1 mg lAA, 0.2 mg NAA, 1.5 mg Kinetin, 1 mg 6 benzyladenine purine (BAP), 1 mg zeatin and 0.5 mg GA_3/L. On medium containing 3% sugar, 0.2 mg NAA and 2.25 mg BAP, 36.7% of Desiree and 33.3% of Spunta explants formed callus of which 46.7 and 16.7% respectively regenerated.

Smolenskaya (1989) produced primary callus by in vitro culture of potato leaf and stem explants on medium with various supplements. Explant of the primary callus was cultured on the same medium but without benzylaminopurine (BAP) and NAA or both of these growth regulators. The experimental results showed a successful shoot formation on medium with Zibbrallin Bezylaminopurine (BAP) 0.3 mg/L in the medium inhibited shoot formation while presence of Zibbrallin alone (0.5 mg/L) or in combination with NAA (0.03 mg/L) stimulated shoot formation and also rooting which occured a months after subculturing.

Ravinkar and Gogala (1990) observed the in vitro influence of jasmonic acid (JA) and differentiation of meristems of potato cv. Vesna. Meristems were grown on Murashige and Skoog (MS) medium supplemented with IAA (10μM), kinetin (10μM) and GA_3 (3μM). Addition of 0.5-10 μM JA increased the number of meristems that developed into buds, particularly in meristems isolated from shoots grown from tubers in the dark. JA had no noticeable effect on meristems from germs grown in light. All added concentration of JA retarded callus and root formation. The inhibitory effect on rhizogenesis disappeared immediately after transfer of the developed buds to medium without JA.

Martel *et al.* (1992) conducted an experintent with potato cv. Sebago leaf discs were cultured on Murashige and Skoog media with 30 mg sucrose/L + 0-4 mg BA were added to the initial medium and 0-4 mg BA + 1 or 5 mg GA_3 for the shoot proliferation medium. NAA was essential for callus formation. The amount of callus formation increased with the increasing concentration of both NAA and BA, with best result obtained with the highest concentrations of both the growth regulators. Both BA and GA_3 were necessary for shoot formation. Shoot formation occurred more rapidly with higher BA concentrations.

Omidi and Shahpiri (2003) studied the effect of growth regulators (2, 4-D and kinetin), cultivar, explant and light on callus induction in potato. In addition, the effects of cultivars and explants were evaluated on callus organogenesis. At first callus induction on internode explants was tested using MS medium supplemented with combination of 1, 2 or 3 mg/L 2, 4-D and 0.00, 0.01 or 0.10 mg/L kinetin. They found a significant effect of 2, 4-D and kinetin concentration and their interaction, on frequency of callus induction and number of roots on callus.

2.2.3 Shoot regeneration

Smolenskaya (1989) reported a successful shoot formation on medium with zibbrallin, benzylaminopurine (0.3 mg/L) in the medium inhibited shoot formation while presence of zibbrallin alone (0.5 mg/L) or in combination with NAA (0.03 mg/L) stimulated shoot formation and also rooting which occurred a month after subculturing.

The plantlet regeneration in cultivars Chieftain, Desiree, Kennebec, Lenape, Niska, Russet Burbank and Shepody from petioles with intact leaflets was assessed by Yee *et al.* (2001) using six treatment carbonations basal medium with or without silver thiosulphate or thidiazuron at two concentration (2 or 0.5m g/L) of IAA. The basal medium consisted of Murashige and Skoog (MS) salts and vitamins supplemented with 3 mg 6- benzyl aminopurine/L, 1 mg gibberellic acid/L, 30g sucrose/L and 7 g phytoagar/L. They observed that silver thiosulphate decreased the regeneration frequency and number of shoot per callus but no significant changes were observed with thidiazuron. Regeneration rates of (100%) with upto 20 shoots plantlets per callus were achieved at 2 mg IAA/L with Desiree, Kennebacc, Niska and Lenape. These cultivars still showed high regeneration rate 87-90% on media with 0.5 mg IAA/L and good regeneration rates were also achieved by the other three cultivars (48, 50 and 94% for Chieftain, Shepody and Russet Burbank respectively.

2.2.3.1 Effect of genotype variability

Kazakova (1990) regenerated plantlets by culturing internode tissue of five varieties and a hybrid on agar medium. Results showed a variability in regeneration ability among the varieties where the variety Yantarnyi had the best regeneration ability.

Ao and Liu (1991) cultured leaf and stem explants from 6 cultivars on MS media supplemented with various combinations of growth regulators. Plant regeneration frequency averaged 60.1-88.7% for the 6 cultivars reaching 162.3% under the most favourable combination of genotypes and growth regulators.

2.2.3.2 Effect of explant

Alphonse *et al.* (1998) carried out an experiment with leaflet explants, tuber discs and callus tissues from micropropagated plantlets of potatoes cv. Cara, Desiree, Diamant and Spunta.The explants were cultured 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 that from tuber discs was greatest with Diamont followed by Desiree. Supplementation of MS medium with 1 mg/L benzyladenine, 1mg/L IAA and 10 mg/L zibberellic acid (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 the best combination for tuber discs.

In vitro regeneration was assessed by Hamdi *et al.* (1998) in potato cv. 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 (30g/L), NAA (0.02 mg/L), zeatin riboside (2 mg/L) and gibberellic acid (0.02mg/L).

Hansen *et al.* (1999) investigated shoot regeneration on explant from different leaves and leaflets of potatoes cv. Posmo, Folva and Oleva. Explants were excised from glass house grown plants and grown for 6 days on callus induction medium with indole3 acetic acid or 2,4-dichlorophenoxy acetic acid and then transferred to auxin free shoot regeneration medium with gibberelllic acid and 6-benzyladenine or zeatin. By using the optimum combinations and concentrations of plant growth regulators and by excision of explants from particular regions of proximal leaflets from newly unfolded leaves, higher frequencies of plant regeneration were obtained from newly developed unfolded leaves for cv. Posmo while the frequency was very low for cultivar Oleva.

Zel *et al.* (1999) examined petioles, internode and leaf explants in combination with different plant growth regulators especially different concentrations of zeatin riboside (ZR). The results revealled that 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 0.02 mg gibberellic acid/L for 2 weeks and then transferred to a shoot induction medium containing 25 m/L.

Rodriguze *et al.* (2000) described a one step regeneration system using leaf effect of different ratios of auxins and cytokinins, added to MS basal medium (supplemented with 30 g/L sucrose, 0.5 g/L thiamin, 1 mg/L gibberellic acid, 40 mg/L ascorbic acid and 1.7 g/L phytagel and a pH of 5.7) was investigated. All leaf explants from DC treated with zeatin riboside (3 mg/L) and indole 3 acetic acid (IAA) 1 mg/L and all leaf explants from PP treated with zeatin ribosite (3 mg/L) induced regeneration, producing green and morphologically normal plants.

2.2.3.3 Effect of growth regulators

Hulme *et al.* (1992) investigated the regeneration of plants from leaf explants of a number of potato cultivars using a number of published one, two and three step methods. A method using a pretreatment with high levels of auxin and cytokinin coupled with silver thiosulphate in the regeneration medium proved to be the most rapid and efficient for the eight cultivars examined.

Yadav and Sticklen (1995) investigated two step procedure used for plantlet regeneration from leaf strips (2-3 mm wide) derived from in vitro grown plantlets of cv. Bintje. Step- I media (M_1-M_6) consisted of MS medium and 9 µM 2, 4-D supplemented with 2.2 µM kinetin (kin), BA, zeatin (Z) or zeatin riboside (ZR). Step-II media (R_1–R_4) consisted of MS medium and 5.78 µM gibberellic acid (GA_3) supplemented with 2.28 µM, BA, Z or ZR. Leaf explants cultured on M_3 or M_6 medium (containing, Z or ZR respectively) for 6 days and then sub cultured on R_4 medium (containing ZR) produced numerous shoots without callus formation. The highest percentage of explants producing shoots (97.5±2.2) and number of shoots/explant (33.7±8) was obtained when M_6 followed by R_4 media were used.

Eshna-Ashari and Villiers (1998) studied plant regeneration from tuber discs of potato (*Solanum tuberosum* L.) using 6 benzylaminopurine (BAP) as a supplement with (MS) basal medium and observed that callus was formed in MS medium with 1mg/L BAP+0.5 mg/L 2,4-D, callus and roots were formed in MS with1 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 1mg/L BAP without the addition of 2, 4-D.

Ehsanpour and Jones (2000) reported one, two and three step methods of plant regeneration from stem culture of potato cv. Delware. Results showed that the one step procedure using thidiazuron, a synthetic cytokinin, was the best for rapid

plant regeneration. In this culture medium, several buds and shoots were regenerated from stem culture, while the other methods using a culture medium supplemented with combinations of GA, BAP, NAA, zeatin, zip (iso-p entenyladenine) and IAA produce white and green callus.

Wang and Song (2000) showed that adding 4 mg $AgNO_3$/L to plant differentiation medium increased the regeneration frequency of Luna- 1from 40% (without $AgNO_3$) to 81%, suggesting that $AgNO_3$ plays an important role in improving plant regeneration in vitro. Another factor affecting regeneration was abscisic acid (ABA). Regeneration frequency was increased by adding 0.2 mg ABA/L to the medium, but high concentrations of ABA (>1 mg/L) inhibited plant regeneration. Genotype, seedling age and type of explant also affected plant regeneration from leaf discs.

Asma *et al.* (2001) studied the effects of different concentration (10, 2.0, 3.0, 4.0 and 5.0 mg/L) of GA_3 (gibberellic acid) and benzylaminopurine (BAP) on the in vitro multiplication of nodal fragments and stem segments of potato cv. Desiree. The maximum shoot length (8.96cm) was obtained when 4 mg GA_3/L was applied. The number of nodes was not significantly affected by any of the GA_3 concentration used in this study. The maximum number of shoots (14) was obtained when 2 mg BAP/L was applied.

Zarnan *et al.* (2001) conducted an experiment with the effect of three different auxins viz. NAA, IAA and IBA each at four levels (0, 0.1, 0.5 and 1.0 mg/L) was evaluated on meristem culture of potato (*Solanum tuberosum* L.) for production of virus free plantlets. Maximum plantlet height (8.3 cm) largest number of nodes/plantlet (7.3) and the highest number of leaves/plantlet (8.9) were recorded at 0.5 mg/L of NAA followed by IBA at 1 mg/L whereas reasonably high number of roots/plantlet (23.7) as well as the earliest microtuber formation (17 days, after

transplation) were recorded at 1mg/L of IBA followed by NAA at 0.1 mg/L Largest root (4.2 cm) was observed for IAA at 1 mg/L concentration.

Ghaffoor *et al.* (2003) conducted an experiment with the effect of three different growth regulators viz. Naphthalene Acetic Acid (NAA), Indole Acetic Acid (lAA) and Indole Butyric Acid (IBA) each at live concentration levels (0.0, 0.05, 0.15, 0.25 and 0.35) were evaluated on meristem culture of potato (*Solanum tuberosum* L.) for production of virus free plantlets. The parameters included were plantlet, height, number of nodes/plantlet, number of leaves/plantlet, root length and number of roots/ plantlet. Maximum plantlet height (9cm) with NAA at 0.15 m/L higher number of nodes/plantlet (9.714) with IBA at 0.35 mg/L and maximum number of leaves/plantlet (6.143) with IAA at 0.25 mg/L were recorded.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present research work was carried out in tissue culture laboratory of the Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur, during the period of December 2009 to April 2010.

3.1 Experimental materials

3.1.1 Plant materials

Three standard potato varieties namely Diamant, Cardinal and Patrones were collected from BADC, Domer, Nilphamari.

3.1.2 Growth regulators

Different growth regulators and their levels were as follows:

IAA (mg/L)	BAP (mg/L)	NAA (mg/L)
0, 0.1, 0.5 & 1.0	0, 1.0, 3.0 & 7.0	0, 0.01 and 0.1

3.1.3 Culture media

Culture media is an important factor for tissue culture. Nutrient media for plant tissue culture are designed to allow plant tissues to be maintained in a totally artificial environment. Here MS (Murashige and Skoog, 1962) medium was used with different hormonal supplements. The composition of MS medium has been listed in Appendix-I. Culture media with different supplements that were used in the present experiment depending on specific purposes are mentioned below.

3.2 Preparation of culture media

Stock solutions were prepared by measuring and dissolving macronutrients. To prepare MS medium, four different stock solutions were prepared. (i) Major salts

(10X concentrated), (ii) Minor salts (100X concentrated), (iii) Iron (100X concentrated) and (iv) Organic nutrients except sucrose (100X concentrated). Each of the needed growth regulators was dissolved separately and kept in bottle. All the prepared stock solutions were labeled properly and kept in refrigerator at 4° C.

3.2.1 Different steps of media preparation

i) Each of the stock solution was pipette into 2 liter Erlenmeyer flask on a magnetic stirrer.

ii) About 500 ml distilled water was added in a flask for dissolving the ingredients.

iii) Myoinositol (100 mg) was added directly to the solution and was dissolved.

iv) Sucrose (30 mg) was added to the solution with proper agitation.

v) Different combinations and concentrations of growth regulators were added to the solution as required and mixed well.

vi) The mixture was then poured into a 1 liter measuring cylinder and made the volume up to the mark with the addition of distilled water and poured back to a 2 liter conical flask and mixed well.

vii) The pH of the medium was adjusted to 5.8 with pH meter with the help of 0.1N NaOH and 0.1N HCl as necessary.

viii) After adjusting pH, about 9 gm agar was added to solidify the medium. The medium was then gently heated with continuous stirring till complete dissolution of agar.

ix) The medium was then transferred to a 1 liter volumetric flask, and made up 1 liter with distilled water.

x) Required volume of hot medium was dispensed into culture vessel viz. test tube or conical flask. Alter dispensing the medium the culture vessels were plugged with non-absorbent cotton and marked with different codes, with the help of a permanent maker to satisfy hormonal combinations.

3.3 Sterilization

All the instruments relevant to *in vitro* culture, glassware, and culture media were properly sterilized using different sterilizing agents.

3.3.1 Sterilization of culture media

The media in the bottles with all necessary ingredients were autoclaved at 1.16 kg / cm^2 of pressure at 121°C for 30 minutes. The medium was then poured into sterile Petri dish and sterile culture vessels in a laminar airflow cabinet and were allowed to cool before use. All the Petri dishes and vials were marked with permanent marker to indicate specific treatment.

3.3.2 Sterilization of glassware and instruments

Petri dishes were wrapped with aluminum foils, vials were capped with plastic caps, cotton, distilled water, metallic instrument like forceps, scalpels, needles etc. were bagged with brown paper and these glassware and instruments were sterilized in an autoclave at a temperature of 121° C for 30 minutes at 1.16 kg/cm^2 pressure.

3.3.3 Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all floors and walls with a detergent followed by wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals.

All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet. The cabinet was switched on for half an hour with ultraviolet light before use and cleaned with 70% ethyl alcohol to reduce the chances of contamination. The instrument like scalpel, forceps, needles etc. were flamed and cooled inside the inoculation chamber before use. Aseptic conditions were maintained during each and every operation to minimize contamination.

3.4 Precautions to ensure aseptic conditions

The essence of aseptic technique is the exclusion of invading microorganisms during experimental procedures. All incubation and aseptic manipulations were carried out under laminar airflow cabinet. The cabinet was usually switched on half an hour before use and wiped with 70% ethyl alcohol to reduce the chances

of contamination. The instruments likes scalpels, forceps, needles etc. were pre sterilized by autoclaving and subsequent sterilization was done by dipping in 70% ethyl alcohol followed by flaming and cooling method inside the laminar flow cabinet. While not in use, the instruments were kept inside the laminar flow cabinet. Hands were also sterilized by wiping with 70% ethyl alcohol. Other required materials like distilled water, glass plate, petridishes etc. were sterilized in an autoclave following the method of media sterilization. The neck of the culture vessels was flamed before opening and closing it with the cap. Aseptic conditions were followed during each operation to avoid the contamination of cultures.

3.5 Explant and size of explants

As explant epicotyl, the internodal parts which are referred to as epicotyl (8 – 10 mm long) from in vitro plantlet were cut under clean hood and inoculated one piece in each test tube for callus induction. For plantlet regeneration, 40 days old calluses were taken out of the test tube and put on the sterilized petridishes and cut as 3 – 4 mm cube under the clean hood. The excised callus pieces were then inoculated into test tube contained 10 ml semi-solid culture media.

3.6 Culture method

3.6.1 Incubation of culture

The explants were prepared with extreme care under the aseptic condition inside the laminar air flow cabinet. One explant was incubated to each test tube containing MS medium supplemented with different hormone concentrations. The test tube was covered and sealed with parafilm.

3.6.2 Culture incubation environment

The cultured dishes were kept in the culture room on the shelves in controlled environment. The test tubes are incubated under complete darkness at 25±2^{0}C for a week after which incubates was subjected to a photoperiod regime of 30μ mol $m^{-2}s^{-1}$ luminance provided by white florescent tubes (4.83 ft). These tubes gave

broad spectrum of light, especially in the red wavelength. The room was illuminated 16 h daily with a light intensity of approximately 1500 lux.

3.6.3 Subculture of the calli for shoot induction

Two weeks after inoculation of explants the calli attained convenient size (20-25 mm), they were removed aseptically from the vial on a sterilized glass plate inside the laminar air flow cabinet and were placed again on freshly prepared medium containing appropriate hormonal supplements for shoot induction from the cells.

3.6.4 Preparation of pot and transplantation

Potting mixture containing garden soil, sand and cow dung in the ratio 1:2:1 was mixed properly and autoclaved one hour in 121°C for 30 minutes at 1.16 kg / cm^2. After cooling, the soil mixture was taken into 10 cm plastic pots for growing the plantlets at *in vivo* condition.

When the plantlets became 7-8 cm in height with sufficient root system, they were taken out from the vials. Medium attached to the roots was gently washed out with running tap water. The plantlets were then transplanted to pot containing potting mixture mentioned 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. The interior of the polythene bags was sprayed with distilled water at every 24 hrs to maintain high humidity around the plantlets. At the same time, plantlets were also nourished with Hogland's solution. After two to three days the polythene bags were gradually perforated to expose the plants to natural environment. The polythene bags were completely removed after ten to fifteen days when the plantlets appeared to be self- sustainable. At this stage, the plantlets were placed in natural environment for 3-10 hours daily. Finally, after 15-20 days they were transferred to the field condition

3.7 Recording data

To investigate the effect of different treatments and response of different varieties to callus induction and plant regeneration, data were collected on the following parameters.

3.7.1 Callus induction

a. Days required for callus induction

Generally callus initiation started after fourteen days of incubation of explants. The number of callus initiated over a number of days was recorded. The mean value of the data provided the days required for callus initiation.

b. Percent callus formation

The percentage of callus formed was calculated using the following formula

$$\% \text{ of callus formed} = \frac{\text{No. of cultures formed callus}}{\text{Total number of cultures}} \times 100$$

c. Weight of callus (mg) and rate of growth

The callus weight was calculated 25 DAI (Days after incubation) and the growth rate of callus was calculated by using the following formula

$$\text{Growth rate of callus in weight (mg/day)} = \frac{\text{Weight of callus in mg}}{\text{Total period (40 days)}}$$

3.7.2 Shoot induction

a. Days to shoot induction

When the shoot regeneration was started the data were recorded.

b) Percent of shoot development

The percentage of shoot development were calculated by using the following formula

$$\% \text{ of shoot development} = \frac{\text{No. of callus induced shoot}}{\text{No. of callus incubated}} \times 100$$

c. Shooting index

Potential of shooting of callus with different treatments varied widely which was measured by an arbitrary scale 0 – 5, where, 0 = lowest and 5 = highest on the basis of growth rate.

3.7.3 Rooting

a) Days to rooting

The culture was carefully observed for root development and when any root elongation seen was treated as days to rooting.

b) Rooting index

Potential of rooting with different treatments varied widely which was measured by an arbitrary scale 0 – 5, where, 0 = lowest and 5 = highest.

3.8 No. of shoot per culture

Multiple shoots have been found in many cultures, which were counted at 40^{th} day after culture and recorded as number of shoot per culture.

3.9 Plant height at 40 DAC (Days after culture)

The height of plantlets was measured against a centimeter scale. The plantlet was measured from the base to tip. In case of multiple shoot, the tallest plantlet was taken into consideration.

CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

After the placement of epicotyl of potato in MS media supplemented with different growth hormone, callus was derived first, then shoot, root and finally full plantlet. The results obtained in the present research are presented below.

4.1 Callus formation

4.1.1 Callus formation (%)

Maximum percentage of callus formation (78.82%) obtained from epicotyl when cultured on medium containing 0.5 mg/L IAA + 3.0 mg/L BAP. BAP (1-7 mg/L) alone induced callus formation ranging from 35.30 to 50.00% (Table 4.1) while IAA 0.5 mg/L and onwards did not form any callus though 0.1 mg/L IAA formed 46% callus (Table 4.1). Generally, IAA or BAP alone showed low callusing in explants while they together formed more callus. However, comparatively low levels of IAA and BAP found more effective than higher levels. Yeasmin (2002) found maximum (90%) callusing with 1.25 mg/L NAA + 1.0 mg/L BAP. Here, in 0.5 mg/L of IAA and 3.0 mg/L of BAP showed highest result. No callus formation was observed on medium devoid of growth regulators. The varieties did not very significantly for callus initiation. It ranged from 45.75% in Patrones to 48.81% in Diamant (Fig. 1). It was happened due to varietal difference.

The interaction results of these varieties with IAA+BAP on callus formation are presented in Table 4.3. Cardinal showed 90.35% callusing with 0.5 mg/L IAA + 3.00 mg/L BAP. No callus was developed with control and 0.5 or 1.0 mg/L alone. The others varied from 18.68% to 82.87% (Table 4.3).

Table 4.1 Effects of IAA+BAP on callus formation and growth in epicotyl of potato

Treatment (IAA+BAP) mg/L	Callus formation (%)	Days required for callus induction	Weight of callus and rate of growth	
			Callus weight (mg)	Rate of growth (mg/day)
0.0+0.0	0	0	0	0
0.0+1.0	35.30	15.83	555.32	13.88
0.0+3.0	52.42	19.89	2250.25	56.25
0.0+7.0	35.34	21.72	1751.28	43.78
0.1+0.0	48.58	18.21	950.68	23.76
0.1+1.0	70.51	13.52	2418.39	60.45
0.1+3.0	65.87	9.36	2217.98	55.44
0.1+7.0	68.25	11.25	2554.17	63.85
0.5+0.0	0	0	0	0
0.5+1.0	70.51	10.32	3542.40	88.56
0.5+3.0	78.82	11.85	3660.80	91.52
0.5+7.0	58.60	9.87	3578.40	89.46
1.0+0.0	0	0	0	0
1.0+1.0	68.25	8.51	2392.00	59.80
1.0+3.0	70.53	11.73	2995.20	74.88
1.0+7.0	56.36	9.54	3425.20	85.63
LSD 0.05	1.55	3.81	608.25	12.58
CV (%)	18.36	16.25	14.37	19.44

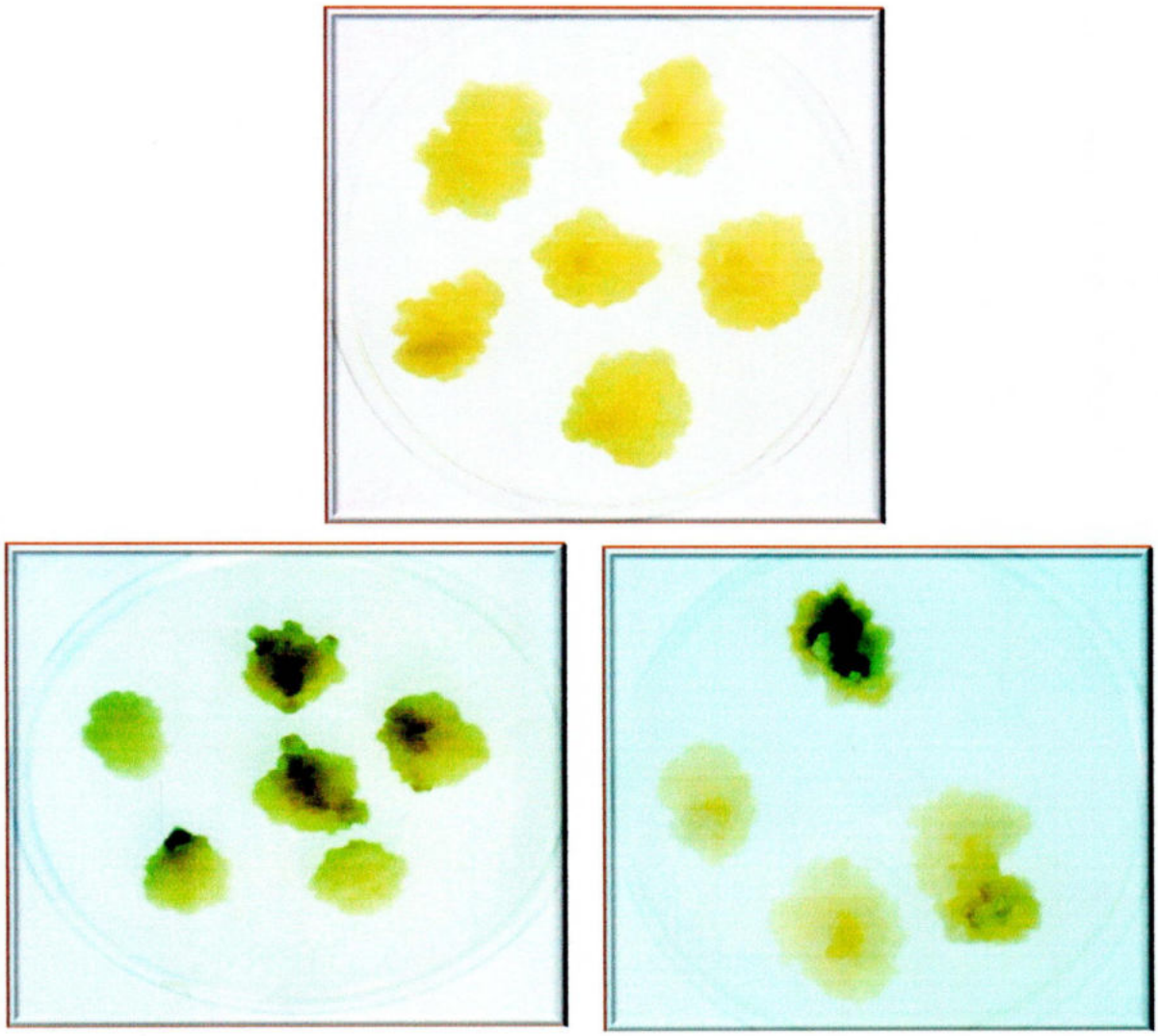

Plate 1: Callus formation in Diamant (top), Cardinal (bottom left) and Patrones (bottom right)

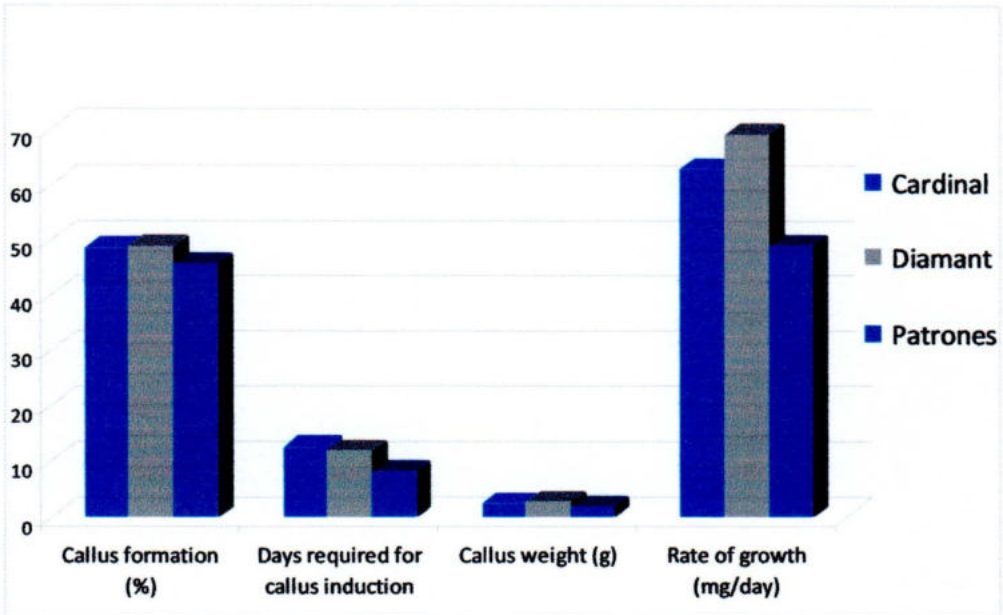

Fig. 1 Varietal difference in calls formation and growth in epicotyl of potato

4.1.2 Days required to callus induction

Significant variation was observed for days required to callus induction. Callus initiated in most of the IAA+BAP treatments in epicotyls within 8-12 days. In case of IAA or BAP alone took more days to initiate callus though the lowest level of BAP (1.0 mg/L) took 15.83 days. Roy (2004) cultured four different potato varieties on MS + 0.5-3.0 mg/L NAA + 0.25-1.0 mg/L BAP and found callusing within 5-6 days which is controversial to this investigation. The difference might be due to the different kind of auxin used. The hormonal combination of the present investigation was (0.0, 0.1, 0.5 and 1.0 mg/L) IAA + (0.0, 1.0, 3.0 and 7.0 mg/L) BAP. Nisa (2003) used different levels of NAA and BAP for callus induction in potato varieties and recorded 8.13 days at 2.5 mg/L NAA + 2.0 mg/L BAP and 7.45 days at 10.0 mg/L NAA alone which show a good match with this investigation.

The callusing ranged from 8.32 to 12.54 days (Table 4.2). The varieties with different IAA + BAP levels showed statistically significant differences on this parameter (Table 4.3). In case of IAA or BAP alone took more days (25.61) for Patrones with 7.0 mg/L BAP (Table 4.3). Lower level of IAA + BAP took more time than the higher level. It was found that BAP was effective in callus formation, which was enhanced in addition of IAA. Roy (2004) found significant difference in Heera, Dheera, chamak and Diamant and in callus formation but here no significant difference was found in Cardinal, Diamant and Patrones.

Table 4.2 Combined effects of IAA+BAP and variety on callus formation and growth in epicotyl of potato

Treatment		Callus formation (%)	Days required to callus induction	Callus weight and rate of growth	
(IAA+BAP) (mg/L)	Variety			Callus weight (mg)	Rate of growth (mg/day)
0.0+0.0	Cardinal	0	0	0	0
	Diamant	0	0	0	0
	Patrones	0	0	0	0
0.0+1.0	Cardinal	35.24	18.50	830.20	20.75
	Diamant	48.54	22.93	1137.29	28.43
	Patrones	18.68	4.20	421.10	10.52
0.0+3.0	Cardinal	43.52	16.97	1645.28	41.13
	Diamant	48.48	17.53	3382.34	84.55
	Patrones	61.37	23.79	1237.83	30.94
0.0+7.0	Cardinal	42.38	21.99	2192.52	54.81
	Diamant	24.26	15.76	1763.73	44.09
	Patrones	35.20	25.61	2020.31	50.50
0.1+0.0	Cardinal	42.87	22.63	587.39	14.68
	Diamant	42.58	10.30	729.58	18.23
	Patrones	48.48	20.60	1545.34	38.63
0.1+1.0	Cardinal	82.64	13.62	2457.68	61.44
	Diamant	82.87	9.77	3275.81	81.89
	Patrones	68.96	11.37	1848.65	46.21
0.1+3.0	Cardinal	73.20	7.88	2588.54	64.71
	Diamant	68.96	9.21	2210.53	55.26
	Patrones	73.20	7.43	1864.36	46.60
0.1+7.0	Cardinal	68.96	11.46	2578.58	64.46

	Diamant	80.52	9.81	2496.35	62.40
	Patrones	73.20	7.03	1829.27	45.73
0.5+0.0	Cardinal	0	0	0	0
	Diamant	0	0	0	0
	Patrones	0	0	0	0
0.5+1.0	Cardinal	80.52	9.66	3574.84	89.37
	Diamant	73.58	10.54	5047.54	126.18
	Patrones	72.83	8.85	2732.58	68.31
0.5+3.0	Cardinal	90.35	9.63	4526.38	113.15
	Diamant	80.52	11.83	4854.12	121.35
	Patrones	82.35	10.45	2772.83	69.32
0.5+7.0	Cardinal	68.96	9.61	4325.18	108.12
	Diamant	64.52	12.41	4785.46	119.63
	Patrones	55.84	6.81	2698.72	67.46
1.0+0.0	Cardinal	0	0	0	0
	Diamant	0	0	0	0
	Patrones	0	0	0	0
1.0+1.0	Cardinal	80.52	8.04	4023.36	100.58
	Diamant	72.83	10.58	2756.97	68.92
	Patrones	68.25	9.04	2572.31	64.30
1.0+3.0	Cardinal	72.83	9.74	3485.16	87.12
	Diamant	81.26	8.44	5213.65	130.34
	Patrones	72.83	11.24	2154.36	53.85
1.0+7.0	Cardinal	55.51	6.57	3521.15	88.02
	Diamant	72.83	9.22	5350.25	133.75
	Patrones	72.83	9.71	2685.15	67.12
LSD		2.68	6.70	1080.35	21.05
CV (%)		15.56	17.74	16.85	16.85

4.1.3 Weight of callus and rate of growth

IAA + BAP highly influenced the callus weight. Both these hormones together gave higher callus than that with IAA or BAP alone (Table 4.1). 3660.80 mg was found as maximum callus growth with 0.5 mg/L IAA + 3.0 mg/L BAP and the growth rate was 91.52 mg/day. The minimum was 555.32 mg and growth rate was 13.88 mg/day with 1.0 mg/L BAP. BAP alone gave the heavier callus than from IAA. While they together gave 4-6 times heavier callus than that with either IAA or BAP. Roy (2004) cultured nodal section of potato variety Diamant with 0.5-3.0 mg/L NAA + 0.25-1.0 mg/L BAP and recorded maximum 0.287 mg size callus which was contradictory to the results of present investigation.

Callus weight and growth rate was significantly different among the varieties. Diamant had biggest (2764.35 mg) callus with higher growth rate (69.10 mg/day) followed by Cardinal (2515.69 mg and 62.89 mg/day growth rate). Patrones was found as the poorest. Roy (2004) recorded 156-202 mg callus after 30 days of culture which did not match with the results of the present investigation. Interaction of variety and IAA + BAP showed maximum callus weight of 5350.50 mg and growth rate 133.75 mg/day by Diamant when cultured with 1.0 mg/L IAA + 4.0 mg/L BAP. The minimum weight of callus was 421.10 mg and 10.52 mg/day growth rate for Patrones for 1.0 mg/L of BAP. In most of the IAA+BAP levels, Diamant performed best and Patrones was least, that means the used levels was not suitable for Patrones as that of Diamant and Cardinal (Table 4.2).

4.2 Shoot development

4.2.1 Days to shoot induction

Shooting appeared in a range of 1.65 to 13.33 days (Table 4.3). Either BAP or NAA alone took more time to develop shoot. Shoot development was minimum of 6.28 days for Diamant, which was statistically similar to Patrones (7.11 days). Cardinal was very

late to form shoot (11.23 days) (Fig. 2). All the three varieties with BAP and NAA showed a wide range of variation in shooting.

4.2.2 Percent of shoot development

Low levels of BAP+NAA was found to enhance the shoot development. Shooting was lowest (2.03%) with 2.0 mg/L BAP + 0.0 mg/L NAA. Maximum 57.04% shooting occurred for 1.0 mg/L BAP + 0.01 mg/L NAA (Table 4.3).

Maximum 37.60% shoot developed in Patrones followed by Cardinal (29.91%). Diamant showed the least (23.62%). This seems to be due to varietal difference. Shooting in callus culture of different varieties with BAP and NAA varied significantly.

Table 4.3 Effects of BAP+NAA on shoot and root development from callus of potato

BAP+NAA (mg/L)	Days to shoot induction	Percent of shoot development	Shooting index
0.0+0.0	1.65	7.32	0.35
0.0+0.01	10.18	38.27	1.95
0.0+0.1	13.33	57.04	4.02
1.0+0.0	8.56	14.76	0
1.0+0.01	11.25	53.19	4.68
1.0+0.1	8.32	38.17	4.25
2.0+0.0	2.54	2.03	0
2.0+0.01	8.43	24.71	4.42
2.0+0.1	2.87	9.91	1.50
LSD	2.93	1.43	0.69
CV (%)	20.82	19.78	18.01

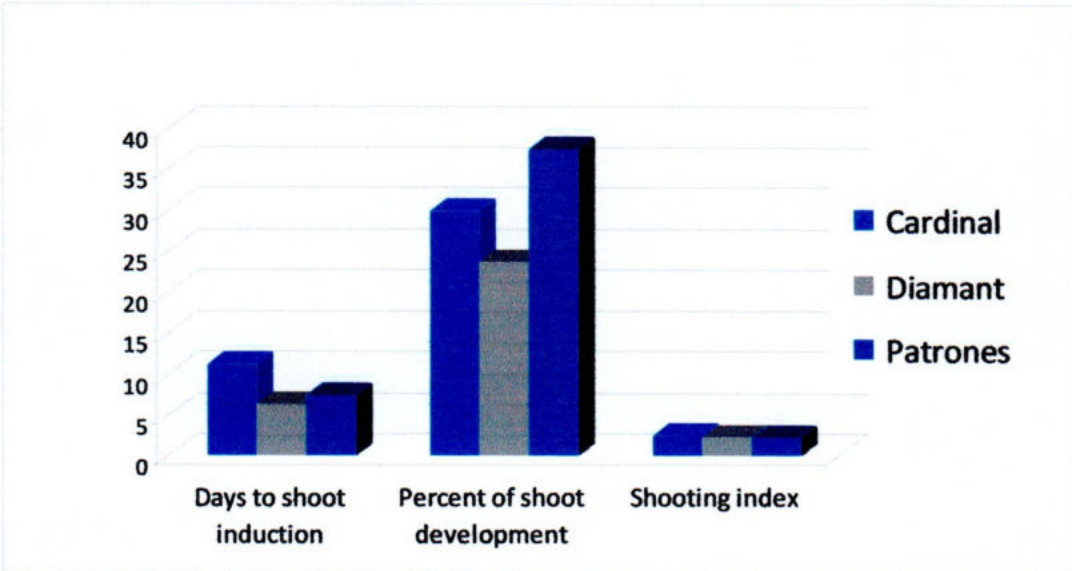

Fig. 2 Effect of variety on shoot and root development from callus of potato

Table 4.4 Combined effects of BAP+NAA and variety on shoot and root development from callus of potato

Treatment		Days to shoot induction	Percent of shoot development	Shooting index
BAP+NAA (mg/L)	Variety			
0.0+0.0	Cardinal	4.15	18.43	0.37
	Diamant	0	0	0.40
	Patrones	0	0	0.35
0.0+0.01	Cardinal	14.35	43.47	2.30
	Diamant	10.26	34.27	2.10
	Patrones	12.74	48.24	2.22
0.0+0.1	Cardinal	13.24	33.65	4.15
	Diamant	15.18	67.52	4.02
	Patrones	14.36	55.38	4.22
1.0+0.0	Cardinal	20.54	0	0
	Diamant	0	0	0
	Patrones	12.64	48.21	0
1.0+0.01	Cardinal	14.32	55.31	5.00
	Diamant	15.37	43.84	5.00

	Patrones	9.65	55.59	4.48
1.0+0.1	Cardinal	13.27	53.66	4.45
	Diamant	12.55	35.53	4.35
	Patrones	8.52	42.45	4.28
2.0+0.0	Cardinal	7.26	0	0
	Diamant	0	0	0
	Patrones	0	0	0
2.0+0.01	Cardinal	13.27	33.26	4.82
	Diamant	8.58	18.55	4.65
	Patrones	7.94	17.87	4.55
2.0+0.1	Cardinal	5.26	11.14	1.30
	Diamant	0	0	1.25
	Patrones	7.12	18.64	1.30
LSD CV (%)		5.08 18.78	2.49 17.01	1.20 16.90

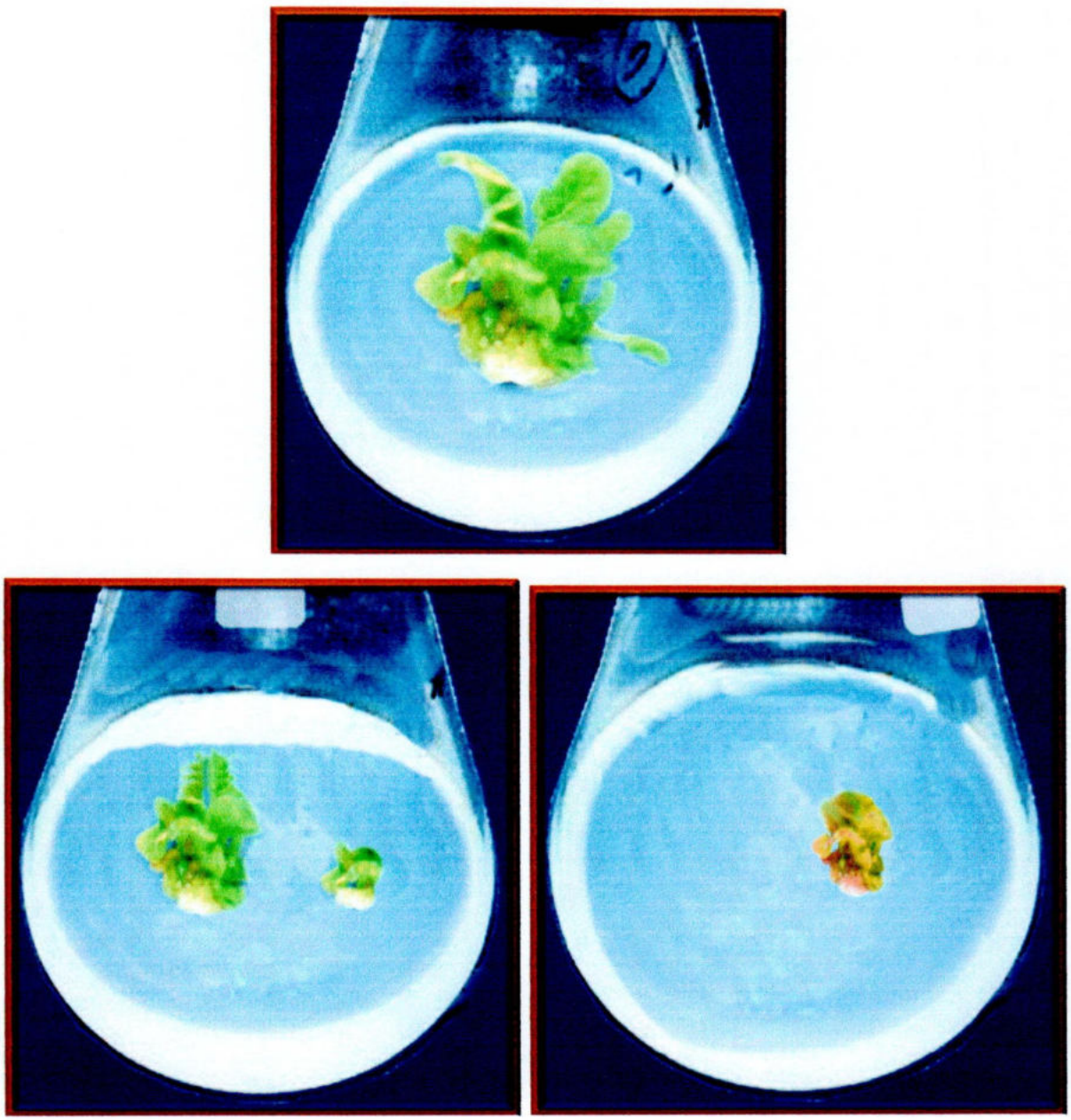

Plate 2. Shoot formation in Diamant (top), Cardinal (bottom left) and Patrones (bottom right)

4.2.3 Shooting index

Shooting index varied significantly with the treatment. Maximum (4.42) shoot index was obtained with 2 mg/L BAP and 0.01 mg/L NAA while minimum was 0.35 with the control (Table 4.2). BAP at together with 0.01 mg/L or 0.1 mg/L NAA showed better result of shooting index.

No significant difference was found among the varieties. Cardinal and Diamant showed better with 1.0 mg/L BAP and 0.01 mg/L NAA. Patrones showed lower shooting index with either BAP or NAA alone (Table 4.4).

4.3 Root formation

4.3.1 Days to rooting

Rooting did not develop with control or with 1.0 or 2.0 mg/L BAP (Table 4.5). Earlier root development was found with 2.0 mg/L BAP and 0.1 mg/L NAA (7.94 days) which was statistically similar to a number of treatments. The treatment 1.0 mg/L BAP + 0.01 mg/L NAA was inferior and required 11.24 days (Table 4.5). NAA alone formed roots at 10.55 to 12.33 days in subcultures from callus and BAP alone formed no roots.

Cardinal introduced to root formation after 6.48 days, which was superior to other varieties. Diamant and Patrones were statistically similar and took 8.75 and 7.36 days for rooting respectively (Fig. 3). Most delayed root formation was found in Patrones after 12.33 days. NAA was used in culture media which was statistically similar to four treatments. No variety formed root without growth regulators of even with 1 or 2 mg/L BAP alone. It indicates that BAP has no effect on root formation (Table 4.6)

Table 4.5 Effects of BAP+NAA on the growth and development of root and shoot from potato callus

BAP+NAA (mg/L)	Days to rooting	Rooting index	No. of shoot per culture	Plant height (cm)
0.0+0.0	0	0	0.10	0.40
0.0+0.01	10.55	4.01	1.20	2.48
0.0+0.1	12.33	4.30	1.36	3.02
1.0+0.0	0	0	2.01	4.52
1.0+0.01	11.24	3.45	1.25	3.98
1.0+0.1	9.52	3.65	1.20	3.75
2.0+0.0	0	0	1.67	4.78
2.0+0.01	8.87	3.36	1.25	4.12
2.0+0.1	7.94	3.25	1.75	4.25
LSD	1.08	0.28	0.39	0.64
CV(%)	17.28	16.78	19.01	20.52

4.3.2 Rooting index

NAA highly influenced the rooting potential of cultures. Rooting index was highest for Diamant (2.35) while Cardinal (2.25) and Patrones (2.15). Cardinal and Patrones were statistically similar and inferior. Rooting index was 4 in many treatments, while it was drastically reduced with 2 mg/L BAP. Lower NAA levels found more effective in developing vigorous roots quickly (Table 4.6).

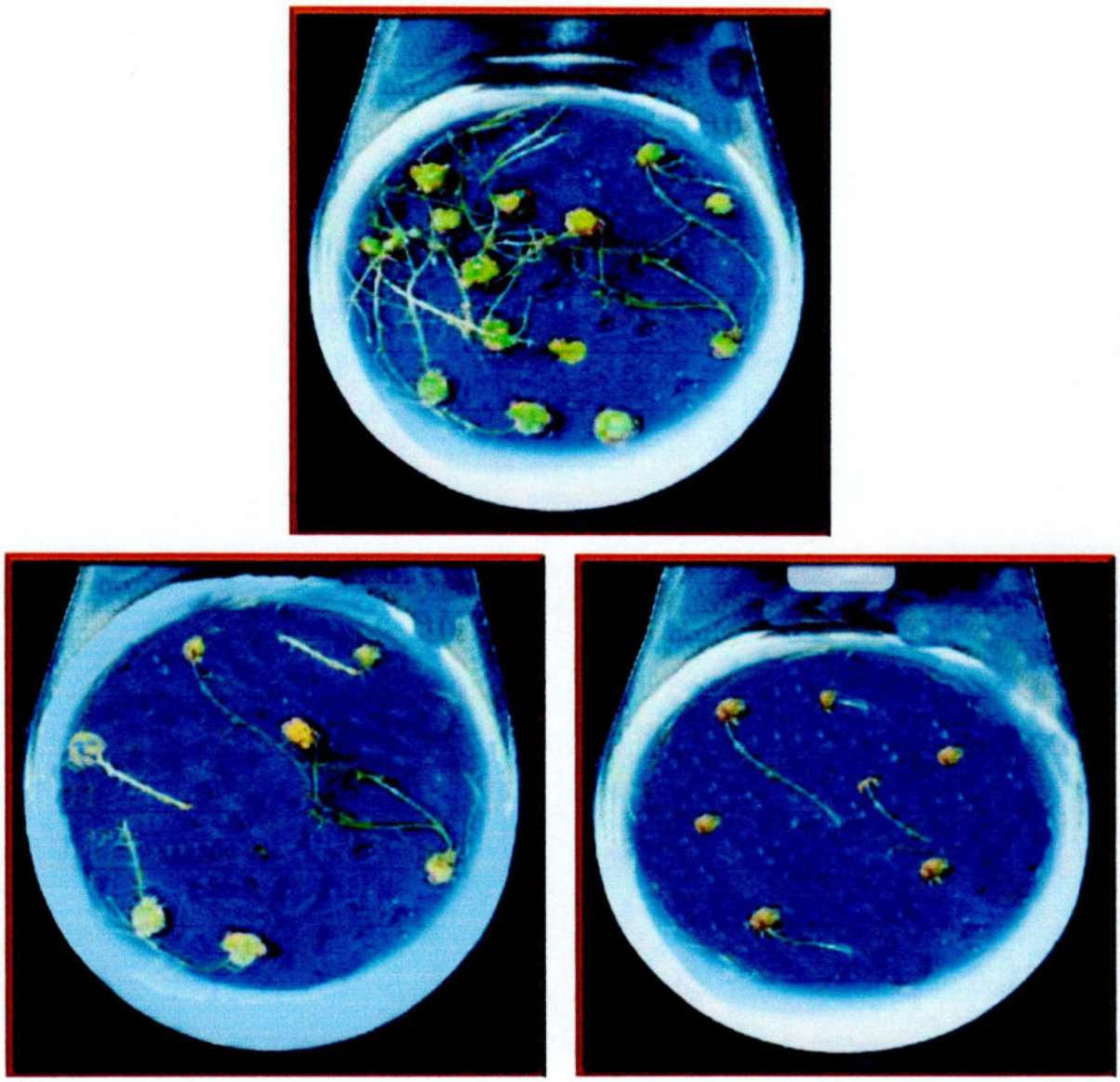

Plate 3. Root formation in Diamant (top), Cardinal (bottom left) and Patrones (bottom right)

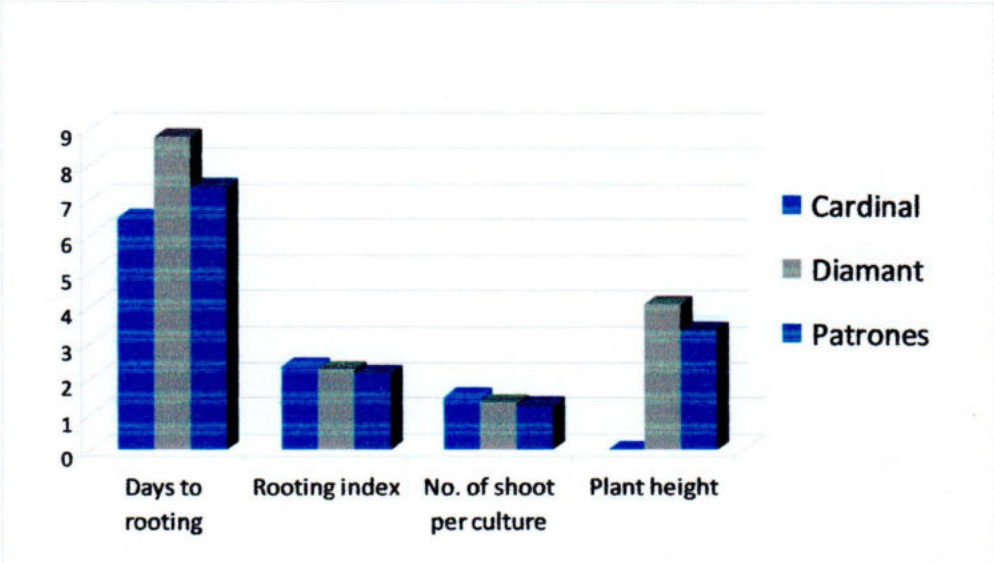

Fig. 3 Effect of variety on the growth and development of root and shoot from potato callus

4.4 Number of shoot per culture

Developed plantlet formed more than one shoot (Table 4.5). Maximum number of shoot was 2.01 for 1.0 mg/L BAP. Most treatments with BAP alone or in combination with NAA formed better shoot, which proved BAP as a good shoot promoter. Varieties did not differ significantly for number of shoots per culture. The varieties with BAP+NAA showed significant results on the parameters. The variety Cardinal formed maximum of two shoots per culture when only 1.0 mg/L BAP used, which was statistically similar to many treatments (Table 4.6). Roy (2004) obtained maximum 8.6 shoots per culture by adding 2.0 mg/L BAP + 1.0 mg/L GA_3 + 1.0 mg/L Kinetin, which did not match with the result of the present investigation.

Table 4.6 Combined effects of BAP and NAA and variety on the growth and development of root and shoot from potato callus

Treatment		Days to rooting	Rooting index	No. of shoot per culture	Plant height (cm)
BAP+NAA (mg/L)	Variety				
0.0+0.0	Cardinal	0	0	0.20	0.48
	Diamant	0	0	0	0
	Patrones	0	0	0	0
0.0+0.01	Cardinal	10.75	3.48	1.30	2.54
	Diamant	13.24	4.01	0.85	1.57
	Patrones	12.31	4.10	1.15	2.94
0.0+0.1	Cardinal	8.95	3.50	1.64	3.15
	Diamant	13.28	4.28	1.15	3.12
	Patrones	14.25	4.35	1.18	2.54
1.0+0.0	Cardinal	0	0	2.01	4.51

	Diamant	0	0	1.91	4.95
	Patrones	0	0	1.31	5.03
1.0+0.01	Cardinal	10.76	3.42	1.65	4.48
	Diamant	12.56	2.58	1.15	4.42
	Patrones	14.98	3.00	1.64	3.52
1.0+0.1	Cardinal	9.28	3.26	1.68	4.87
	Diamant	14.03	3.51	1.18	4.12
	Patrones	12.87	2.75	1.62	3.28
2.0+0.0	Cardinal	0	0	1.67	5.07
	Diamant	0	0	1.88	5.13
	Patrones	0	0	1.40	5.53
2.0+0.01	Cardinal	12.58	3.92	1.42	5.49
	Diamant	12.75	2.52	1.60	4.38
	Patrones	11.45	2.43	1.44	3.47
2.0+0.1	Cardinal	10.38	2.75	1.65	6.15
	Diamant	15.37	3.02	1.83	4.02
	Patrones	10.01	2.75	1.62	4.05
LSD		1.87	0.49	0.67	1.11
CV (%)		17.28	14.10	19.68	17.29

4.5 Plant height (cm)

Plant height was significant varied due to addition of BAP + NAA in the culture media. A plant height of 0.2 cm was for the control treatment (Table 4.5). Maximum plant height was 4.78 cm for 2 mg/L BAP (Table 4.5) closely followed by 1 mg/L BAP (4.52cm). This result again proved the positive effect of BAP of shoot development.

Plant height varied significantly among the varieties. Diamant showed maximum height of 4.08 cm and the minimum was for Cardinal (3.10cm) (Fig. 3). It was due to varietal difference.

4.6 Hardening and transplantation

Potting mixture containing garden soil, sand and cow dung in the ratio 1:2:1 was mixed properly and autoclaved one hour in 121°C for 30 minutes at 1.16 kg / cm^2. After cooling, the soil mixture was taken into 10 cm plastic pots for growing the plantlets at *in vivo* condition.

When the plantlets became 7-8 cm in height with sufficient root system, they were taken out from the vials. Medium attached to the roots was gently washed out with running tap water. The plantlets were then transplanted to pot containing potting mixture mentioned above. At this stage, the plantlets were placed in natural environment for 3-10 hours daily. Finally, after 15-20 days they were transferred to the field condition. The survival rate of plantlet was 70%.

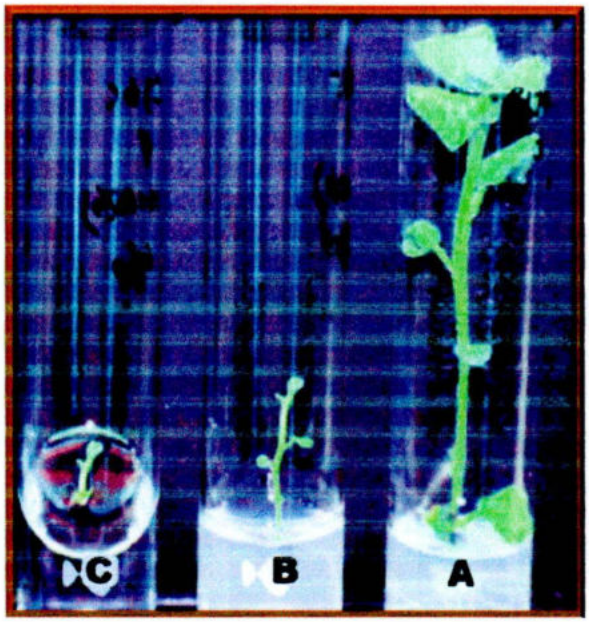

Plate 4. Plantlets of Diamant (A), Cardinal (B) and Patrones (C)

Plate 5. An established plant in pot

CHAPTER V

SUMMARY AND CONCLUSIONS

CHAPTER V

SUMMARY AND CONCLUSION

The experiment in relation to callus induction, shoot formation, root initiation and plantlet regeneration of three standard potato varieties were conducted at the tissue culture laboratory, Department of Genetics and Plant Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur during the period of December 2009 to April 2010.

For callus induction, epicotyl isolated from *in vitro* microplants of varieties Cardinal, Diamant and Patrones were cultured on MS media containing four levels of IAA (0, 0.1, 0.5 and 1.0 mg/L) and BAP (0.0, 1.0, 3.0 and 7.0 mg/L). The developed calluses were sub-cultured to regenerate plantlet on MS media containing three levels of BAP (0.0, 1.0 and 2 mg/L) and NAA (0.0, 0.01 and 0.1 mg/L). All these studies were conducted during the period of December 2009 to April 2010. The final findings of this experiment are presented below:

BAP (0.0, 1.0, 3.0 and 7.0 mg/L) alone induce callus up to 50% cultures. Maximum 78.82% callusing was obtained with 0.5 mg/L IAA and 3.0 mg/L BAP. No callus was formed in medium without hormone or with 0.5-1.0 mg/L IAA. No significant difference was found among the varieties under this parameter. The varieties with the IAA+BAP levels showed maximum 90.35% callusing in Cardinal with 0.5 mg/L IAA + 0.3 mg/L BAP. While 18.68 to 82.35% callus initiated in other treatments. IAA or BAP alone took more time to start callusing in epicotyl. All the varieties with either IAA or BAP alone had delayed callusing (15-20 days) while 0.1 mg/L IAA and 1.0 mg/L or 3.0 mg/L or 7.0 mg/L BAP took 8-12 days for callus induction.

Highest callus weight obtained with IAA+BAP. IAA or BAP alone formed lower weight of callus (555.32 mg). Heavier callus was 3660.80 mg with 0.5 mg/L IAA + 3.0 mg/L BAP and the growth rate was 91.52 mg/day. Among the varieties Diamant showed highest callus weight (2764.35 mg). Results with all the varieties with IAA and BAP showed that Diamant had heavier callus (5350.25 mg), and the rate was 133.75 mg/day with 1.0 mg/L IAA and 7.00 mg/L BAP.

Maximum 57.04% callus formed shoot directly with 0.1 mg/L NAA or 1.0 mg/L BAP and 0.01 mg/L NAA. Interaction of NAA and BAP with varieties showed maximum 67.52% shoot development in sub-culturing of callus in Diamant with 0.1 mg/L NAA.

At lower levels of BAP and NAA, it took more days to direct shooting while early shooting appeared with minimum days of 6.28 in Diamant and most delayed in Cardinal (11.23). All the varieties showed shooting with minimum days with highest levels of BAP and NAA. Shooting index varied from 0.35 to 5 across the BAP and NAA treatments. It appeared that 1.0 mg/L BAP and 0.01 or 0.1 mg/L NAA exhibited maximum shooting index. Cardinal and Diamant with 1.0 mg/L BAP and 0.01 mg/L NAA had maximum 5.0 shooting index.

Rooting in culture appeared within 7.94 to 11.24 days across the treatment while the control and 1 or 2.0 mg/L BAP did not developed any root. Rooting index varied from 3.25 to 4.30 across the BAP+NAA treatments. All the varieties with NAA alone or BAP+NAA levels showed a rooting index >4.0.

The number of shoots per culture ranged from 0.10 for control to 2.01 for 1 mg/L BAP. The variety Cardinal with 1.0 mg/L BAP developed maximum 1.5 shoots per culture.

Plant height ranges from 0.40 to 4.78 cm. 4.78 cm was obtained from 2.0 mg/L BAP alone. Among the varieties plant height was highest in Diamant (4.08 cm) and lowest in Cardinal (3.10 cm).

It can be concluded that epicotyl of *in vitro* microplants of potato variety Cardinal, Diamant and Patrones can effectively be used for callus induction using the best combination of 0.5 – 1.0 mg/L IAA and 3.0 – 7.0 mg/L BAP. Among the varieties the plantlet regeneration was highest with 1.0 mg/L BAP + 0.01 or 0.1 mg/L NAA.

The outcome from the present investigation may help in the large scale production of potato from epicotyl as an explant. Although the varieties were used here, further investigation can be done to know the potentiality of those varieties through biotechnological approaches.

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