2002

Improvement of Potato (Solanum Tuberosum L.) Through in Vitro Culture

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IMPROVEMENT OF POTATO (*Solanum tuberosum* L.) THROUGH *IN VITRO* CULTURE

A THESIS SUBMITTED TO
THE UNIVERSITY OF RAJSHAHI IN FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF PHILOSOPHY

IN

BOTANY

2002

By

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DEDICATION

I dedicate this thesis to my beloved
father, Late Abdur Razzaque
and
mother, Lutfun Nesa
who encouraged me through the years

CERTIFICATE

It is my pleasure to certify that the thesis entitled "IMPROVEMENT OF POTATO (*Solanum tuberosum* L.) THROUGH *IN VITRO* CULTURE" is submitted by **Khandker Din Mohammad** to the University of Rajshahi, Bangladesh for the degree of Master of Philosophy in Botany.

I hereby certify that the author completed his work fully under my direct supervision, and to the best of my knowledge no materials of this thesis previously published or written by any other person except, wherever, due references are made in the text of the thesis.

Rafiul Islam 19.10.2002

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DECLARATION

I hereby declare that the entire research work submitted as a thesis for the Degree of Master of Philosophy in Botany at the University of Rajshahi is the result of my own investigation.

Khandker Din Mohammad 19.10.02

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Candidate

ABSTRACT

In order to develop disease free potato seeds *in vitro* culture was established using meristems of potato cultivar, Diamont. Meristems were isolated from 25-30 days old field grown plants and cultured onto filter paper bridge in liquid MS medium. Among 29 media formulations medium with MS+0.1 mg/l KIN + 0.5 mg/l GA_3 was found to be the best media formulation for primary establishment of meristem culture. Estabilished meristems were subcultured onto agar solidified medium. Among 22 medium formulations media with 0.5 mg/l BAP + 1.0 mg/l IBA was found to be the best for shoot proliferation. For all four potato cultivars *viz.* Diamont, Cardinal, Multa and Lalpakri sucrose was found to be the best carbon source and 3% sucrose was better than 6% sucrose for shoot proliferation.

For adventitious root induction medium with MS+0.05 mg/l IBA + 0.05 mg/l GA_3 was appropriate media composition among 16 media formulation and 3% sucrose was best among seven concentrations (10-70 g/l) of sucrose for root induction for all four cultivars of potato.

Maximum *in vitro* tuberization was obtained in media supplemented with 8 mg/l KIN among six concentrations (2-12 mg/l) of BA and KIN. Medium contained 60 g/l sucrose was found to be the best for microtuber induction for all four cultivars among various concentration (10-70 g/l) of sucrose. Continuous photoperiods (24 hour) produced more microtubers than 0 and 8 hour photoperiods.

Comparative field evaluation of potato plants obtained from three propagule sources *viz.* tuber, microtuber, and microshoot indicate that tuber yield of tuber produced plants was higher than microculture (microshoot, microtuber) produced plants. Between microshoot and microtuber produced plants tuber yield of microshoot produced plants was higher than microtuber produced plants. But the tuber number of microculture produced plants was greater than the plants obtained from tuber. Disease reaction indicates that microculrture produced plants showed few contamination by virus, bacteria and fungus than the tuber produced plants.

The results of the present study showed that disease free potato seeds could be developed through meristem culture which may help to increase potato production in Bangladesh.

ACKNOWLEDGEMENT

All my acknowledge and gratitudes to the Almighty Allah for countless blessing which he showered on me to make up this research programme.

It is dept of gratitude and pleasant due to express my obligation and personal indebtedness to my respected teacher and research supervisor Dr. A.K.M. Rafiul Islam, Professor, Department of Botany, University of Rajshahi, Bangladesh for his kind and constant guidance, cordial behavior and untiring perseverance throughout the progress of this research work and preparation of this thesis. I am grateful for his intellectual, potential supervision and valuable advice.

I offer heartiest gratitude to the affectionate and assistance given by Dr. M. Firoz Alam, Associate Professor, Dept. of Botany, University of Rajshahi, in the preparation of this manuscript.

I like to extend my cordial thanks to Dr. M. Monzur Hossain, Professor, Department of Botany of the same University for offering valuable instruction in this assignment.

I also wish to express my sincere gratitude to Professor N.K. Paul, Chairman, Department of Botany, University of Rajshahi for granting academic facilities to carryout my research works in this department.

I am extremely grateful to Professor, M. Nurul Amin, Professor, M. Shah Alam, Professor, M Anisur Rahman, Professor, Asheq Ahamed, Dr. M. Shahidul Alam, Mr. M. Khalequzzaman, Mr. M. Zahangir, Mr. M. Anisuzzaman, Assistant Professors of the same Department for their kind help benevolent inspiration, encouragement and suggestion during preparing this manuscript.

I would like to mention the co-operation of my lab. members, Mr. Iftakher Ahamed, Hassanur Rahman, Mrs. Joly Alam, Sujon, Tutul, Masud, Minu, Nuruzzaman and Rana who have cordially co-operated me in various ways throughout my research work.

My thanks also due to M.A. Khalack and Salim Saduger (Babu) for their tireless efforts in typing this manuscript.

Finally, I express appreciation and gratefulness to my beloved parents, uncle, Dr. Moshihur Rahman, brothers, K.M. Humayun Reza Kabir, K.M. Nabiul Alam and wife, Nahid Sultana who encouraged and kept me out of domestic worries throughout the study. Not only those thanks are also to all my well wishers.

Khandker Din Mohammad

Abbreviations of the special words used in the text

BAP	6-Benzyl amino purine
NAA	α-naphthalene acetic acid
KIN	6-Furfuryl amino purine (kinetin)
2,4-D	2,4-dichlorophenoxy acetic acid
IAA	Indole-3-acetic acid.
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
MS	Murashige and Skoog (1962) medium
pH	Negative logarithm of hydrogen ion concentration
mg/l	Milligram per litre
g/l	Gram per litre
$HgCl_2$	Mercuric chloride
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
KOH	Potassium hydroxide
O.1N	O.1 Normal
Roxb.	W. Roxburgh
sp./spp.	Species
et al.	*et ali* = Other people
e.g.	exempli gratia = For example
viz.	Videliect = Namely
cm	Centimetre(s)
°C	Centigrade(in degree)
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
etc.	et cetera = And the others
g	Gram(s)
i.e.	Id est = That is
%	Percentage
No.	Number
Fig.	Figure (S)

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INTRODUCTION

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INTRODUCTION

The necessity of self-sufficiency in food and feeding stuff is vital for the economic well being in a country. One way this need could be satisfied by performing the extensive research work for the improvement of economically important crops that exist in the country. Potato is one of the important crops grown for its tuber value in making several processed items and in vegetables. To make Bangladesh self-sufficient with respect to vegetables, plant breeders are trying to improve the virus free clones through *in vitro* technique and modern culture technology.

1.1 BOTANICAL ASPECT OF POTATO :

Potato (*Solanum tuberosum* L.) is an important food crop in Bangladesh and all over the world. It is one of mankind ancient cultivated plant and ranks fourth in food production, following wheat, maize and rice. Potato crop is produced in a greater quantity than any other grain crop. They are marked as an easy digestible and provides vitamins (B&C), starch, protein, iron, magnesium, potassium and amino acids. At present, potato is grown in about 133 thousand hectares in Bangladesh and annual production is 1468000 tones in 1995.

Day by day potato is being more popularized in Bangladesh in producing various delicious food and dry food product. Potato was unknown most of areas of the world before 400 years. Before 6000 B.C. in South America, Gypsi Red Indian people collected potato from the plants which grown naturally on the 12,000 ft height of Andis. Day by day they began to cultivate the potato. In 16^{th} century, by the Spanish intruders it spread throughout continental Europe and parts of Asia. From England, it spread to Ireland, Scotland, Wales and parts of northern Europe Singh (1989).

In 1719, the people from Ireland migrated in U.S.A. and they introduced potato there first. In 1715 Portuguese firstly introduced potato in Indian sub-continent from Europe, and became popular there.

1.2 ORIGIN AND TAXONOMIC POSITION OF POTATO :

Potato originated from the southern part of Peru and Andis's region of Bolivia. In 16th century Spanish brought it to Europe from South America. In 17th century potato came in Indian sub-continent.

Potato (*Solanum tuberosum* L.) was originated from the cross of cultivate diploid variety *Solanum stenotorum* and wild type diploid variety *Solanum sprsipilum*. It is a segmental polyploids (Hawkes, 1978).

In the world, potato has eight cultivable species and more than one thousand wild species. Among these species *Solanum tubrosum* is most widely used and most popular in the world. It is the member of Solanceae family. It is annual and dicotiledonous plant. Its height is about 45 to 200 cm.

Solanaceae family has 85 genus and 2000 species. Lots of important crops and plants are the members of Solanceae family, such as potato, eggplants, tomato as vegetables, chilli as spice, tobacco as smoke, *Atropa belladona* and *Datura metel* as medicine, *Cestrum nocturnum* and *Petunia hybrid* as flower and *Physalis peruviana* as fruits.

The potato belongs to a single species, (*Solanum tuberosum* L.) a part in certain cultivated forms in South America. In addition to *S. tuberosum,* seven cultivated species and 154 wild species of potato are generally recognized. The rest of the genus consists of non-tuberiferous species such as *S. nigrum* and many spiny herbs and shrubs. The tuber-bearing species are consisted to the American continent and many are of considerable interest to potato occurs, because of their resistance to pests and pathogens and their adaptation to the extremes.

TAXONOMIC POSITION :

Kingdom	:	Plant kingdom
Sub kingdom	:	Phanerogamia
Division	:	Angiosperm
Class	:	Dicotyledoneae
Order	:	Solanales
Family	:	Solanaceae
Genus	:	*Solanum*
Species	:	*tuberosum*

1.3 SYSTEMATIC OF THE GENUS *SOLANUM* :

The genus *Solanum* L. is extremely large, containing about 2000 species (Hawkes, 1944). But only some 150-200 species in this vast genus are tuber bearing. The tuber bearing species belong to the sub-genus *Pachystemounum*, Section *Tuberaruim*, Subsection, *Hyperbasarthrum*. Within the subsection *Hyperbasartrhrum*, Hawkes (1944) recognized thirteen series, twelve of which had been previously differentiated by Bukasov (1939). The cultivated potatoes, including *Solanum tuberosum*, belong to these thirteen series.

1. *Juglandifolia* Rydb. (Colambia, Ecuador, North Peru) not tuber bearing; rather distinct from the other series.
2. *Conicibaccata* Bitt. (Mexico, Central America, Colombia, Ecuador, Peru, Bolivia) tuber bearing.
3. *Etuberose* Juz. (South central Chile, also in Argentina) not tuber bearing.
4. *Bulbocasatana* Tydb. (Mexico, Guatemala) tuber bearing.
5. *Cardiophyll* Buk. (Mexico) tuber bearing.
6. *Pinnatissecta* Tydb. (USA, Mexico, Guatemala) tuber bearing.
7. *Commer soniana* Buk. (Argentina, Uruguay, Brazil, South Bolivia) tuber bearing.
8. *Acaulia* Juz. et Bud. (Peru, Bolivia, Northern Argentina) tuber bearing from high altitudes and have marked frost-resistance.
9. *Demissa* Buk. (Mexico) tuber bearing widely used in breeding for *Phytophthora* resistance.
10. *Longipedicellate* Buk. (Mexico, Southern U.S.A) tuber bearing.
11. *Cuncolate* Hawkes (Desert regions of South Bolivia, Northern Chile) tuber bearing.
12. *Polyadenia* Buk. (Mexico) tuber bearing.
13. *Trberoca* Rydb. (Confined to S. America, almost entirely to the Andis and their immediate surroundings) tuber bearing. The cultivated potatoes, including *Solanum tuberosum*, belong to these series.

1.4 POTATO VARIETIES:

The varieties grown in this country can be categorized into (a) Modern Potato Varieties (MPV) and (b) Indigenous Potato Varieties (IPV). In general, the varieties introduced here in the recent past, particularly since early sixties, are known as modern varieties and the varieties which were introduced long before from different parts of the world and became degenerated and have lost their identify, but are still under cultivation under different popular names are known as indigenous varieties. The Indigenous Potato Varieties (IPV) are also known as local or 'Deshi' potato varieties. The name of some indigenous varieties are (i) Lalpakri (ii) Shilbilati (iii) Jhaubilati (iv) Hera (v) Dohazari (vi) Surjyamukhi (vii) Shadaguti (viii) Patnai (ix) Challisha etc.

Modern varieties are very much popular for high yielding. Lots of modern varieties are introduced in Bangladesh from India and Holland. List of the varieties from Holland are (i) Cardinal (ii) Diamont (iii) Baraka (iv) Binaz (v) Altimus (vi) Yarla (vii) Multa etc. List of the varieties from India are (i) Kufri chandramukhi & (ii) Kufri jyoti.

The recommended modern varieties are (i) Cardinal, (ii) Diamont, (iii) Kufri sunduri (iv) Multa (v) Petronese (vi) Lalpakri (vii) Jhaubilati etc.

1.5 ECOLOGY:

The potato has a wide range of seasonal adaptability. It is a cool season crop and is moderately tolerant to frost. It thrives in cool regions where there is sufficient moisture and fertile soil. The ideal soil for potato is one which is well drained, well aerated, deep and having a pH range 5.2 to 6.4. So, potato can be produced on a wide range of soils, ranging from sandy loam, silt, loam and caly soil. Well drained sandy loam and medium loam soils, rich in humus are most suitable for potato. Alkaline or saline soils are not suitable for potato cultivation. They are well suited to acidic soils (pH 5.0 - 6.5) as acidic conditions tend to limit scab disease. (Singh, 1989). Satisfactory tuber growth occurs at 17°C and 19°C but at about 30°C and above the tuber production is totally stopped.

1.6 MORPHOLOGY :

Tuber : Potato is a herbaceous annual plant. It is vegetatively propagated by means of tuber. The tuber is an enlarged underground stem produced on the end of a stolon and not on the roots proper. The tubers are morphologically stems.

Stem : The upper part of sprout develops in to the aerial stem. The aerial stems are round or angular, pubescent or glabrous, green or purplish. The stems are branching type.

Leaves : The leaves of potato are alternate and compound (occasionally bicompound).There are three to four pairs leaflets arising in succession along the rachis. The leaflets are more or less opposite.

Root : The root of potato is adventitious, arising from the base of a sprout. The root growth is usually restricted to top layers, at a depth of about 20-25 cm from soil surface.

Flower : The flowers of the potato plant are in terminal clusters. Each flower contains five stamens, two celled pistil, five sepals and five petals united for about half their length. The colour of the corolla varies from blue to nearly white and is a distinguishing varietal characters.

1.7 DISEASES OF POTATO IN BANGLADESH:

In vegetative propagated potato crops, once systematically infected with a viral diseases, the pathogen is passed from one vegetative generation to next. The entire population of a given variety may, over years be infected with the same pathogen. The indigenous varieties are playing an important role of potato production in Bangladesh. But since their introduction, the varieties have now became permanently infected with different viruses. Khan (1981) reported that a single plant of potato variety has been infected with four or five viruses. Moreover, potato has a very little defense against viral disease. Viral diseases are the most serious disease infecting potato in Asian countries and results substantial losses of yield and deterioration of tuber quality of the potatoes. Virus free clone may be

developed especially by using meristem culture. The presence of viral disease is an important reason attributed to low yield of these varieties (Ahmed, 1981; Siddique and Hussain, 1988; Liu and Li, 1996). The yield reduction may be up to 75% caused by the infection of some virus (Rashid, 1987).

The identified viruses are (i) PLRV (ii) PVX (iii) PVY (iv) PVS (v) APMV (vi) ALAV (vii) PSTVD (viii) PYDN (ix) APMV (x) ALAV (xi) PSTVD (xii) PYDN.

All most all of IPVs are contaminated with virus such as PVS, PVM, PVY, PVX, PAVM, and PLRV (Potato Leaf Roll Virus) and yellow disease caused by mycoplasma. Most of the IPV seed potatoes used by the growers are extremely poor quality in respect of size and virus infection. Crops raised from such tubers generally do not perform well and produce a high proportion of plants showing the symptoms of "yollowing" caused by mycoplasma. Consequently the yields obtained are very low. The low yield of the IPVs may also be associated with some diseases (primarily viral) carried with the tubers (Kim *et al.*, 1996). The improvement of IPVs have not been given proper attention by the different research organizations. Tuber Crop Research Center (TCRC) of BARI is mainly concerned with research for the improvement of modern potato varieties (MPVs). TCRC conducted different aspects of research for the improvement of IPVs. However, the out come of the research does not reach to the farmer level. During 1983 - 84 BADC in collaboration with Bangladesh Agricultural University (BAU) under the Dutch technical assistance strengthen the research for improvement of IPVs. The research program included optimization of proper doses of fertilizer, selection of disease free plants, cleaning yellow disease, use of insecticide and selection and use of bigger size tuber as (28 - 35 cm size) seeds. The yield of some IPVs have been increased considerably using these cultural practices. Recently, a virus cleaning program through tissue culture of IPVs have been started at the Department of Horticulture, Bangabondhu Sheik Muzibur Rahman Agricultural University, Gazipur in collaboration with BAU under the financial assistance of CDP/DAF, but the findings of the research are yet to be come out.

Table 1: Causal agent, infected parts and symptoms of potato diseases.

Name of diseases	Causes	Infected parts	Symptoms
A. Fungal disease			
i) Late blight	*Phytophthora infestans*	Leaves, stems and tuber	The symptoms are brown to black, fast spreading leaf spots, drying of leaves and stem and white mildew in the underside of leaves.
ii) Early blight	*Alternaria solani*	Leaves and tuber	The main symptom is the presence of dead spots with concentric ring on leaves, but there is no wilting.
iii) Verticillium wilt	*Verticillium alboatrum*	Leaves and tuber	Verticillium wilt can affect the top leaves first, causing them to turn yellow and eventually die. When this symptom occurs, the entire stem dies quickly. More typically, the lower leaves turn yellow and eventually die. The stem tends to remain erect rather than flopping over when it dies. The eyes of the tubers of the infected plants may be turned to pink.
iv)Rhizocton Canker	*Rhizoctonia solani*	Stem and tuber	Buds are roted, not germinated. Plants wilt. Cottony white, mycellium can be found in infected part.
B. Bacterial diseases			
i) Brown rot	*Pseudomonas solanacearum*	Stem and tuber	The symptoms are wilting, stunting and yellowing of the foliage followed by collapse to the plant. There is brrowning of the xylem in the vascular bundles. The skin of the infected tubers is discoloured.
ii) Black leg	*Erwinia sarotovora*	Tuber, leaves and stem	Stems of infected plants typically have inky black. Leaves turn yellow and leaflets tend to roll upwards at the margin of leaflets and later entire plants may wilt and eventually die. The flesh of the tubers is cream colored, gradually turning to grayish and finally black.
iii) Bactarial ring rot	*Corynebacterium sepedonicum*	Leaves, stem and tuber	The first symptoms of the disease usually appear late in the growing season as a pale yellow green mottling on the lower leaves which may be restricted to a single stem or found on numerous stem of the hill. The edges of these leaves eventually curl upward and start to die. The symptoms progress up the stem and eventually the entire stem wilts, turns yellow and dies. The symptoms in the tuber is a yellowish white cheesy appearance in the vascular ring followed by milky, creamy ooze which may be forced out of the tuber is squeezed.
iv) Common scab	*Streptomyces scabies*	Tuber	Rough brown spots of tuber is the main symptom of this disease.
C. Viral diseases			
i) Leaf Roll	Potato leaf roll virus (PLRV)	Leaves, tuber	Rolled, upwards, leathery texured and light green coloured. Tuber size is small and a few number.
ii) Mild Mosaic	Potato virus 'A' and 'X' (PAXV)	Leaves	When only virus 'A' is present, light green gouges leaves are formed. There may be some dead spots on the foliage and distinct mottling and crinkling of leaves.
iii) Rugose mosaic or veinbanding mosaic	Potato virus 'Y' and 'X' (PYXV)	Leaves and stem	The affected plant remains stunted, the leaves are severely wrinkled and sometimes mottled. Necrotic streaks may be present over the leaves and stems. The petioles and leaves become brittle and plants ultimately die.

1.8 POTATO BREEDING :

Bangladesh is predominantly an agricultural country. Various kinds of crop species are fitted into the agriculture system of Bangladesh. Among them rice, jute, sugarcane, potato and tea are main cash crops. Potato is the major winter crop. Additionally some tuber and vegetables are also grown here. The improved yield potential (25%) of cultivated crop like potato has only been obtained through conventional breeding progamme. Further increase of about 20-25% has been possible also due to improve cultural practices. However, conventional breeding has potential limitation (Chu, 1986; Cutter, 1978; Choudhury, 1994; Wriedt, 1985).

Potato breeding has a wide range of yield improvement. Now several plant breeders are working in Bangladesh, which has a distant advantage of promoting breeding technique. Among the different breeding programms, tissue culture is a recent establishment for potato in the Dept. of Botany, University of Dhaka and also being pursued in BRRI, Bangladesh Agricultural University (BAU), Bangladesh Atomic Energy Commission (BAEC), Bangladesh Institute of Nuclear Agriculture (BINA), Chittagong University (CU), Rajshahi University (RU), Jahangir Nagar University (JU), and BRAC.

Single shoots regeneration and production of multiple shoots of potato have been possible through *in vitro* culture. Efforts have been made on rapid propagation through meristem culture in *Solanum tuberosum*. For potato, process has been standardized for clonal propagation through meristem culture. Mass propagation through *in vitro* culture of potato is also in progress. Experiment on somaclonal variants are now progressing well in different somaclones obtained from different potato varieties. Somaclonal variants with useful properties have been produced in potato (Choudhury, 1994).

Tissue culture technique in breeding programme has also been successfully applied for obtaining meristem culture of potato. Application of tissue culture technique for micropropagation of potato and other crop plants seems highly potential with great success, met in this regard in the Department of Botany, R.U. Good success has been achieved in multiplying potato species through tissue culture. Using shoot tip and nodal segment also successful micropropagation of different species of potato have been made.

At the Horticulture Department of Bangabandhu Sheik Muzibur Rahman Agricultural University, Gazipur work is in progress on:

a) Microtuber production of indigenous potato varieties.

b) Micropropagation of virus free potato (Choudhury, 1994).

1.9 ROLE OF MICROPROPAGATION FOR THE IMPROVEMENT OF POTATO:

For many years tissue culture has been applied to improve potato production by means of micropropagation, pathogen elimination and germplasm conservation (Roca, *et al.,* 1979; Schilde, 1982). However some of these techniques are still being refined and improved (Espinoza, *et al.,*1984;Dodds, *et al.,*1992). intermediate level technologies such as *in vitro* tuberization (Tovar, *et al*;1985), and embryo and anther culture are having some direct application on germplasm distribution and germplasm improvement (Sonnino, 1984). The most sophisticated technologies such as genetic engineering and protoplast fusion have enormous potential to improve potato production but care must be exercised in the translation of that "potential" into reality. The article will analyse this technology spectrum in relation to its impact on potato production.

In vitro micropropagation:

Micropropagation has been used as a tool in the production of nuclear potato seed stocks. It has all the advantages of multiplying Specific Pathogen Tested (SPT) stocks and breaking the cycle of tuber borne soil organism (Kim *et al.*, 1996). In India micropropagation was started in 1982 with the standardization of mass propagation techniques (Villaman *et al.,* 1996). Wu-Zhushu *et al.*, (1991) standardized the technique of mass propagation using node cutting from callus differentiated pantlets. Later, Lango (1992) reported the effect of a lower concentration of NAA, but without working out on the details of the length and number of usable nodes for extensive multiplication. Lango (1992) reported mass production of plantlets from leaf tissues. El-Shobaku and Ibrahim (1997) observed a wide variation in growth behavior in different potato genotypes with regard to their usable nodes for *in vitro* multiplication of virus free mericlones upon verifying the concentration of phytohormones in the medium.

Micropropagation has been developed as a new technology to put forward as a potential way of propagation as well as increasing genetic variability for plant improvement. It has now important practical application in potato breeding and production of potato varieties. During the last 30 years enormous efforts and researches have been going on for the refinement of tissue culture techniques for the improvement of plants. These techniques have been applied to a large number of important potato varieties in agriculture (Hashem *et al.*, 1990; Hussey and Stacey, 1981).

In vitro germplasm conservation:

A number of tissue culture methods have been applied for conservation of potato germplasm *in vitro*., these include the use of growth retrding compounds (Westcott, 1981), reduction of incubation temperature (Schilde, 1979) and less importantly freeze preservation by crypreservation (Grout and Henshow, 1978). Most germplasms apply tissue culture germplasm conservation to some extent; this may be the maintenance of a few genotypes used in a seed programme or it may be a germplasm collection.

Pathogen elimination:

The use of meristem culture has for many years been an important component in potato seed programms. The techniques by producing pathogen tested plants can not only increase marktable yield, but also facilitate gramplasm exchange by removing virus infections; this allows compliance with quarantine regulations. The establishment of pathogen elimination programs has greatly aided the international distribution of potato germplasm and thus indirectly potato production. The use of a wide range of antiviral chemicals included in the culture medium and *in vitro* heat treatment of *in vitro* plantlets should lead to more efficient general pathogen elimination procedures.

In vitro tuberization:

In recent years, interest has developed in many countries on the induction of potato tubers under *in vitro* condition. Several differnt methods are available to bring about the induction process (Tovar, *et al.,* 1985, Estrada, *et al.,* 1986). Potato microtubers produced *in vitro* can be used as a source of germplasm for conservation, transfer between countries, and seed certification schemes. Photoperiod plays an important role in the tuberlization process (Gregory, 1956) and should be optimized in order to enhance tuber size. International Potato Center developed a rapid, cost effective methods that involves the addition of BAP, CCC (chlorocholine chloride) and sucrose to the liquid medium used for propagation (Tovar, *et al.,* 1985)

Genetic Engineering:

One of the great success stories of plant tissue culture was production of developed varieties of potato plants by genetic engineering. In this technique *Agrobacterium* plasmid vectors (Jayen, *et al.,* 1986; Dodds, *et al.,* 1987) are utilized in a system now used by research groups world wide, including laboratories in Europe, the United States, Brazil, Mexico and India. The primary objective of the project is to enhance the nutritional value of the potato by obtaining the supplementary production of a synthetic protein rich in essential amino acids. The synthetic protein is produced by a gene that has been synthesized by machine in the CIP Laboratory.

1.10 A BRIEF REVIEW OF MERISTEM CULTURE :

The tissue culture techniques are grouped into followings four categories on the basis of the plant part used as explant and the type of development *in vitro* (i) embryo culture (ii) meristem culture (iii) anther culture or pollen culture and (iv) tissue and cell culture of all techniques. Meristem culture is unique technique to produce virus free plants.

The cultivation of axillary or apical meristem, particularly of shoot apical meristem is known as meristem culture involves the development of already existing shoot apical meristem and the regeneration of adventitious roots. Meristem cultures have been extensively used for quick vegetative propagation of a large numbers of plant species.

Smith and Murashige (1970) accomplished the first true meristem culture of an isolated angiosperm meristem into complete plant. Before that time it was believed that the isolated shoot special meristem of an angiosperm could not direct its own development but rather, relied on subjacent primordial leaves and stem tissue.

As early as 1993 Porntip independently observed growth of root tips on mineral solutions supplemented with sugars, aspergin and peptone. Zhang (1993) was able to subculture TMV infected tomato roots *in vitro*. Dissecting such roots, and testing the various zones by inoculation of a local lesion host of virus, he noticed that the virus concentration in the terminal parts was low as compared to that of based parts and in the root tips he found no evidence of virus at all.

Limasset and Cornvet (1949) observed that in systematically infected plant virus concentration decreased gradually as they approached to the apical meristem. In the apical meristem no virus was detectable in half of the cases. This led Morel and Martin (1952, 1955) to postulate that it might be possible to isolate the apical meristem of a systematically infected plant *in vitro* in order to obtain virus free plants, genetically identical to the "mother plant".

In the beginning of meristem culture the nutrient medium was based on that developed by White *et al.* (1953), to which minor elements were added according to Robbani (1996). Since then many improvements in the media have been made. A useful basic medium is the one devised by Murashige and Skoog (1962). Increased concentrations of macronutrients were found to be critical for the culture of meristem tips of potato which developed into plantlets more vigorous than that on the previously used low concentration of macroelements (Morel and Martin, 1955).

In most plants in addition to the terminal bud, lateral axillary buds are available which may also be used for meristem tip culture. According to Hollings and Stone (1968) the apical meristem of *Chrysenthemum* gave better survival. Several workers have experienced seasonal influence on meristem tip culture process. Stone (1968) observed better survival of carnation meristem tips in early spring and early autumn than in winter and summer. Van Overbeek (1964), stated that isolated carnation meristem tip produced a high percentage of virus free plants. It may be

concluded that in those instances where viruses are difficult to eradicate by meristem tip culture, the application of heat treatment prior to excision of the explants should be considered. This combined procedure may also be advantageous when as with carnation, only small explants developing slowly and in a low proportion produce virus free plants. In such instances preheat treatment allows the excision of 1-2 mm large tips, rather than 0.1- 0.3 mm meristems, with better chances of growth as leading to virus free plants (El-Fiki *et al.*, 1992).

Morel and Martin (1955) were the first to use meristem culture to eradicate viruses from potatoes in the two decades since, the technique has been widely applied in many countries, including USA, France, Britain, Netherlands, Denmark, Germany, Finland, Japan, Italy and Canada. Virus free clones are now available for most important cultivers. Morel and Martin (1952, 1955) also showed that certain virus could be eliminated from potato and *Dahlia* hybrid by aseptic culture of stem tip. This method allowed the recovery of healthy plants. He also discovered the rapid multiplication of tropical orchid *Cymbidium* using the apical mersitem culture.

Except virus free stocks meristem culture has also many useful applications in crop improvement, clonal propagation, germplasm exchange and germplasm conservation.

Meristem culture has been extensively used for vegetative propagation of many crops and fruit trees. As it is used for obtaining clones, it has been termed as mericlone, i.e. cloning through meristem. Mericloning is useful in clonal multiplication of vegetatively propagated crops e.g., ginger (*Zingibar* sp.), turmeric (*Curcuma domestic*) etc. and of fruit and timber tress and in the maintenance and quick multiplication of breeding materials.

The commonly used propaglues in most of the clonal crops, particularly in the case of those that do not produce seeds, are rather bulky. Further there is the necessity of careful quarantine. Seedlings in test tubes obtained from apical meritems would prove extremely usefully germplasm exchange of such crops. Such seedlings would be free from pathogen and insects and would be easier to handle. This application is likely to became considerable important in the future. Germplasm conservation in clonal crops particularly in the case of root and tuber crops, and in

trees, presents many problems. Root and tubers loss viability rapidly and the cold storage requires large space and expensive. Freeze preservation of merisems and cells in liquid nitrogen at 196°C has been suggested for the long term preservation of their germplasm. This technique is still in the developing stages.

Table 2. List of viruses have been eliminated from potato.

Plants species	Virus elimination	References
Solanum tuberosum (potato)	paracrinkle virus, virus X	Kassanis (1957)
	virus X, virus S	Quak (1961)
		Yora and Tsuchizaki (1962)
		Morel and Muller (1964)
	virus X, virus Y, virus S	Svobodova (1964)
	virus A, virus X, virus S	Kassanis and Varma (1976)
	virus A, virus X, virus Y	Morel *et al.* (1968)
	virus S, virus M	
	virus X, virus S	Stace-Smith and Mellor (1968)
	virus S	Huth and Bode (1970)
	Spindle tuber	Stace-Smith and Mellor (1970)
	virus X, virus Y, virus S	Mori (1971)
	leaf-rol virus	Sip (1972)
	virus A, virus S	Macdonald (1973)
	virus X, virus S	Pett (1974)
	virus S, virus M	Galzy (1972)

1.11 IMPORTANCE OF MERISTEM CULTURE:

Virus elimination through meristem culture is a popular horticulture practice now a days (Bhojwani and Razdan, 1983). This has been demonstrated in a wide variety of agronomically important plants such as *Pisum, Allium, Brssica* and *Solanum* species (Johri *et al.*, 1980). The credit for the initiation of meristem culture of orchid goes to the late Dr. G. Morel of INRA, France (Morel, 1960). Prior to the meristem culture of orchids, very successful results had already been obtained in

carnations, dahlias and potato (Morel and Martin, 1952; 1955). *Cymbidium* seed and seedling were free from the systematic mosaic virus that infected various parts of mature plants such as roots, bulbs, leaves and flowers. Jesen (1955) believed that the young meristematic regions of plants were either not infected by the virus or the tissue has a defense mechanism towards off the pathogen. Morel (1965) suggested that by using such technique large scale orchid plant production could be standardized, or in roses or carnations. Production of large number of flowers of good quality and colour and for the required period in the year could become feasible. *Cymbidium* meristems differentiated sub-apically, whereas, in *Cattlya* the wound tissue near the leaf base proliferated and give rise to meristematic regions (Paet *et al.,* 1996; Wang, 1992; Champagnat *et al.,* 1966; Champagnat and Morel, 1969; Morel 1971).

Details were discussed regarding the nature of propagation method of obtaining sterile meristem, size of explant, pH of the medium and different environmental condition suitable for cultures (Sowokinos and Varns, 1992; Bertsch 1966; Marston, 1966; 1967; 1969; Marston and Varaurai, 1967; Goh, 1971). Isley (1965) and Russon (1965) explained the techniques and details involved in meristem tissue propagation *in vitro.*

The potato is one of the world most important food crops. In 1990 more than 600 cultivars were listed and the number increases each year. Many cultivars, which were once popular and productive gradually decreased in vigour and cropping capacities before it was recognized that the degeneration was largely due to infected with one or more viruses.

The use of meristem culture technique to produce a virus free plant of King Edward (Kassanis, 1957), the clone was propagated and compared with several other clones which were infected with paracrinkle virus, now known as potato virus M (PVM). The virus free clone produced more vigorous haulm and about 10% higher yield, attributed to more tubers rather than large ones (Bawden and Kassanis, 1965). Kassanis and Varma (1967) excised meristems 0.1 mm long, with or without a leaf primordia and observed that only 20/196 buds developed in to plants but 19 were virus free. Morel and Martin (1955) were the first to use meristem

culture to eradicate viruses from potatoes. The technique has been widely applied in many countries.

Smith and Murashige (1970) accomplished the first true meristem culture of an isolated angiosperm meristem into complete plant. Before that time it was believed that the isolated shoot apical meristem of an angiosperm could not direct its own development into leaves and stem tissue (Ball, 1946; 1960). Generally to establish a virus free plant one can culture the apical dome plus two to four subjacent primordia leaves.

At present times several workers have first heat treated the plant material and then cultured large meristem tips from them. The excised meristem tips are up to 1 mm long with two or three leaf primordia. It is assumed that during heat treatment the virus multiplication is inhibited.

Holling and Stone (1967) reported a high proportion of healthy *Chrysanthemum* recovered from leaf mottings virus by meristem tip culture and heat treatment. Russo and Slack (1998), Asatani (1972) found no correlation between the length of heat treatment and proportion of explants free from virus. Other factors were involved when 100% of explants were free from virus with three cultivars and 98% with one cultivar by meristem tip culture.

The application of meristem culture either to eliminate virus infection in clonal plant or large scale production of asexual seedlings and other horticulture benefits have been discussed in many different publications. (Zang *et al.*, 1993; Naik and Widholm, 1993; Patt and Bollen, 1993; Isley, 1965; Russon, 1965; Jessel, 1966; Gripp, 1966; Jasper, 1966; Lindemann, 1967; Voraurai, 1968; Marston, 1969; Lindemann *et al.*, 1970; Bealle, 1971; Bilton, 1971).

1.12 OBJECTIVES:

From all the points of view, the present investigation was undertaken to establish protocol for production of disease free potato tuber from high yielding indigenous and exotic genotypes through *in vitro* techniques with the following objectives.

1. Standardization of culture media for primary *in vitro* establishment of isolated meristems from field grown potato genotypes.
2. Standardization of suitable media composition for rapid shoot multiplication from established meristerm cultures.
3. Selection of suitable media composition for efficient root induction and root elongation.
4. Selection of a suitable nutrient media composition for production of microtubers.
5. Selection of a suitable photoperiod for production of microtubers.
6. Study of dormancy of microtuber.
7. Acclimatization and transplantation of *in vitro* plantlets and microtubers into soil under net house condition and production of virus free potato seed.
8. Field evaluation of the *in vitro* culture derived plantlets under field condition.

Chapter - 2

MATERIALS AND METHODS

Chapter - 2

MATERIALS AND METHODS

2.1 MATERIALS

To conduct the present investigation following materials and equipments were used.

2.1.1 PLANT MATERIALS:

Shoot tips of potato (*Solanum tuberosum* L.) were used as sources for meristem culture. Shoot tips were collected from 25-30 days old field grown plants. In this investigation four varieties of potato *viz*. Diamont, Cardinal, Multa and Lalpakri were selected.

2.1.2 CHEMICALS:

2.1.2.1 Plant growth regulators

The following plant growth regulators were used in the present investigation.

Auxin: Auxin promotes cell enlargement and root induction.

Indol-3-butyric acid (IBA)

Indol-3-acitic acid (IAA)

α-naphthalene acitic acid (NAA)

Cytokinins: Cytokinins promote cell division and shoot initiation.

6-Benzyl amino purin (BAP)

6-Furfuryl amino purin (KIN)

Gibberellins: Gibberellic acid (GA_3)

2.1.2.2 Sterilant and surfactant

$HgCl_2$ was used as surface sterilizing agent and Tween-80 and savlon (an antiseptic, ACI Pharma, Bangladesh) were used as detergent and surfactant.

All chemical compounds including macro and micronutrients, organic and inorganic acids, sugar, agar, KOH, $HgCl_2$, 70 % ethyel alcohol etc. used in the present study were the reagent grade products of either BDH, England or E-Merk, Germany. The vitamins, amino acids and different growth regulators were the products of Sigma Chemical Company, U.S.A.

2.1.3 OTHER MATERIALS:

The culture vessels such as test tube (150×25 mm), bottle (12×5 cm), measuring cylinders, glass rods, beakers, conical flask (250 ml, 1000 ml), pipettes, pipette pump, paraflim, collon plugs, rubber bands, aluminium foils, cotton, forceps, fire box, marker pen, spirit lamp, needle, sharp blade, stereo-microscope, scissors, various size of forceps, electronic balance, magnetic stirrer, autoclave, pH meter, laminar airflow machine etc. were also used in the present experiment.

2.2 METHODS

Experimental methods used for carrying out this investigation were accomplished through following steps.

2.2.1 PREPARATION OF STOCK SOLUTION FOR CULTURE MEDIA

The first step in the preparation of culture medium was the preparation of stock solutions. Various constituents of the respective nutrient medium were prepared into stock solutions for ready use during the preparation of media for different experiments. As different constituents were required in different concentrations, stock solutions of macro-nutrients, micro-nutrients, organic compounds (vitamins and amino acids) and growth regulators were prepared separately.

2.2.1.1 Stock solution (I-III): Macronutrients

This stock solution was made as 20 times of final strength of the medium in 1000 ml of DW. At first 20 times the weight of each of the major salts required for 1 litre of medium was weighted accurately, dissolved once at a time and sequentially in 750 ml of DW and then final volume was made upto 1000 ml by further addition of DW. The stock solution was filtered through Whatman No. 1 (Whatman Ltd. England) filter paper, to remove all the solid contaminants and solid particles like cellulose dust, cotton etc. The stock was then poured in a clean plastic bottle and was labelled by stock solution 1 (20×), stock solution 2 (20×), stock solution 3 (20×) and stored in a refrigerator at 4-6^0 C.

2.2.1.2 Stock solution (IV-VI) : Micronutrients

The stock solution of IV and V were prepared at 20X like stock solution of macronutrients as described earlier (2.2.1.1.). In case of stock solution IV, requisite amount of $FeSO_4.7H_2O$ and Na_2 EDTA. $2H_2O$ were taken and dissolved separately in 400 ml DW by heating and constant stirring. The two solutions were mixed, the pH was adjusted to 5.5 and DW was added to make up the final volume to 1000 ml and

stored in refrigerator at 4-6^{0}C. This solution must be kept in an amber coloured bottle or covered by black paper.

The rest of the micronutrients (solution VI) were made at 1000X in 1000 ml DW. All components of solution VI were weighed separately and dissolved in 800 ml of DW. Finally, the volume of the solution was adjusted to 1000 ml and after filtering stored at 4-6^{0}C in a plastic bottle.

2.2.1.3 Stock solution (VII): Vitamins and Amino acids

The stock solution was prepared at 100X, dissolved in DW to make the volume 200 ml. After being filtered was poured into clean plastic bottle and stored at 0^{0}C in refrigerator.

2.2.1.4 Stock solution of growth regulators

Stock solution of different growth regulators was prepared separately. Details of the preparation methods of stock solution are given in Table 3.

Table 3. Preparation of stock solution for growth regulators.

Growth regulators	Amount taken (mg)	Dissolved in	Final volume with distilled water (ml)	Strength (mg/ml)
IAA	10	80% ethanol 1 ml	10	1
IBA	10	70% ethanol 1 ml	10	1
NAA	10	0.1N KOH 1 ml	10	1
2.4-D	10	70% ethanol 1 ml	10	1
BAP	10	0.1N KOH 1 ml	10	1
KIN	10	0.1N NaOH 1 ml	10	1
2ip	10	0.1N NaOH 1 ml	10	1
GA_3	20	70% ethanol 1 ml	10	1

To prepare stock solution of any of these growth regulators, 10 mg of powdered growth regulators was taken in a clean test tube and dissolved in required volume of appropriate solvent. The final volume of the solution was then made to 10 ml by adding distilled water. The solution was then poured in to a 50 ml glass reagent bottle and stored at 4-6^{0}C in refrigerator.

2.2.1.5 Sterilent solution

$HgCl_2$ solution at various concentrations generally 0.1% was used for surface sterilisation of plant materials. To prepare 0.1% solution, 0.1 g of $HgCl_2$ was taken in a 0.5 litre bottle and dissolved in 100 ml DW. Freshly prepared $HgCl_2$ was always used and generally $HgCl_2$ solution was prepared one hour before use.

2.2.2 PREPARATION OF CULTURE MEDIA

Appropriate amounts of all the components prescribed for a particular medium (mg/l) according to Appendix 1 were mixed taking the required volumes of stock solutions. The final volume of the medium was made by addition of DW. After adjusting the pH of the medium to 5.7± 0.1 using 0.1N NaOH or 0.1N HCl, 8 g/l Difco Bacto-agar and sucrose (BDH) were added. With the special need of experiments any of these (supplements) condition was varied keeping others as unchanged. Media were then heated under a low pressure to melt the agar and sucrose, growth regulators and other supplements. Twenty to 50 ml of medium were dispensed into culture containers of varying sizes. After plugging the culture vessels with non-absorbent cotton wrapped in chess-cloth, media were sterilised by autoclaving at 121^{0}C for 15 minutes at 1.1 kg/cm^2 pressure and stored in the culture room (not more than a week) for ready use.

2.2.3 CULTURE TECHNIQUES

The following methods were employed in the present experiment for primary establishment of meristem culture, subculture, and maintenance of cultures.

2.2.3.1 Collection and surface sterilization of explants

Shoot tips of 25-30 days old field grown potato plants were used as a source of meristem. The shoot tips of potato were excised with the help of sharp blade and collected in a reagent bottle containing distilled water with few drops of detol and few drops of Tween-20 [Polyoxyethelen (20) sorbitan mono-oleate] and quickly brought into laboratory. Then the explants was washed for 2 or 3 times with gradual change of sterile distilled water.

The materials were transferred to 250 ml sterilized conical flask. Surface sterilization was carried out by dipping materials in 0.1% $HgCl_2$ solution with gentle shaking for 2 – 8 minutes followed by 3 – 5 times washing with sterile distilled water in the front of running laminar air flow cabinet.

2.2.3.2 Inoculation technique

All inoculations and aseptic manipulations were carried out in a laminar-air flow cabinet. The cabinet was switched on for half an hour before use and cleaned with 70% ethyel alcohol to reduce the chances of contamination. All instruments like scalpels, needle, forceps, tiles, petri dishes etc. were covered with aluminum foil paper and sterilized by steam sterilization method. During working time, these were again sterilized by 70% ethyl alcohol dip and flaming method inside the inoculation chamber. To ensure complete aseptic condition, both hands were also wiped by 70% ethyl alcohol.

2.2.3.3 Isolation of meristem

After sterilization, explant materials were laid on the sterile tiles using sterile forceps. Shoot tip was hold in one hand under the sterio-microscsope with the help of a pair of forcep and the immature leaves and leaf primordia were snapped with slight pressure from the needle. Then the exposed meristem tips that appeared as a shiny dome were gently isolated with a sharp blade. After deplugging of culture tubes and a single excised meristem tips were carefully placed on the "M" shaped filter paper bridge of the culture tubes containing liquid MS medium. The neck of the tubes were flamed with spirit lamp and then plugged.

After inoculation, the culture tubes were labeled by glass marker pen, then the culture tubes were ready for incubation.

2.2.3.4 Culture incubation

The inoculated culture tubes were incubated in a growth chamber providing a special culture environment. The tubes were placed on the shelves of a culture environment. The tubes were placed on the shelves of a culture rack in the growth chamber. It may be mentioned specially that, all cultures were grown in the growth chamber illuminated by 40 watts white fluorescent tubes fitted at a distance of 30 - 40 cm from the culture shelves. The cultures were maintained at 25° ± 2°C with light intensity varied from 2000 - 3000 lux. The photoperiod was maintained generally 16 hours light and 8 hours dark. The culture tubes were checked daily to note the morphogenic response of culture explants in different experiments conducted in the present investigation.

2.2.3.5 Subculture

Three to four weeks after primary culture, the explants those showed morphogenic response were removed aseptically from the culture tubes and transferred into tubes containing agar gelled MS medium supplemented with different growth regulators. During inoculation, special care was taken that the explant must touch the medium equally and do not dip into medium. After 30 - 35 days of culture initiation shoots were removed aseptically from the culture tubes and placed on a sterile tiles and cut into convenient (basal end of the shoot) size, and again transferred into test tubes containing the same or different growth regulators supplemented in semisolid MS medium.

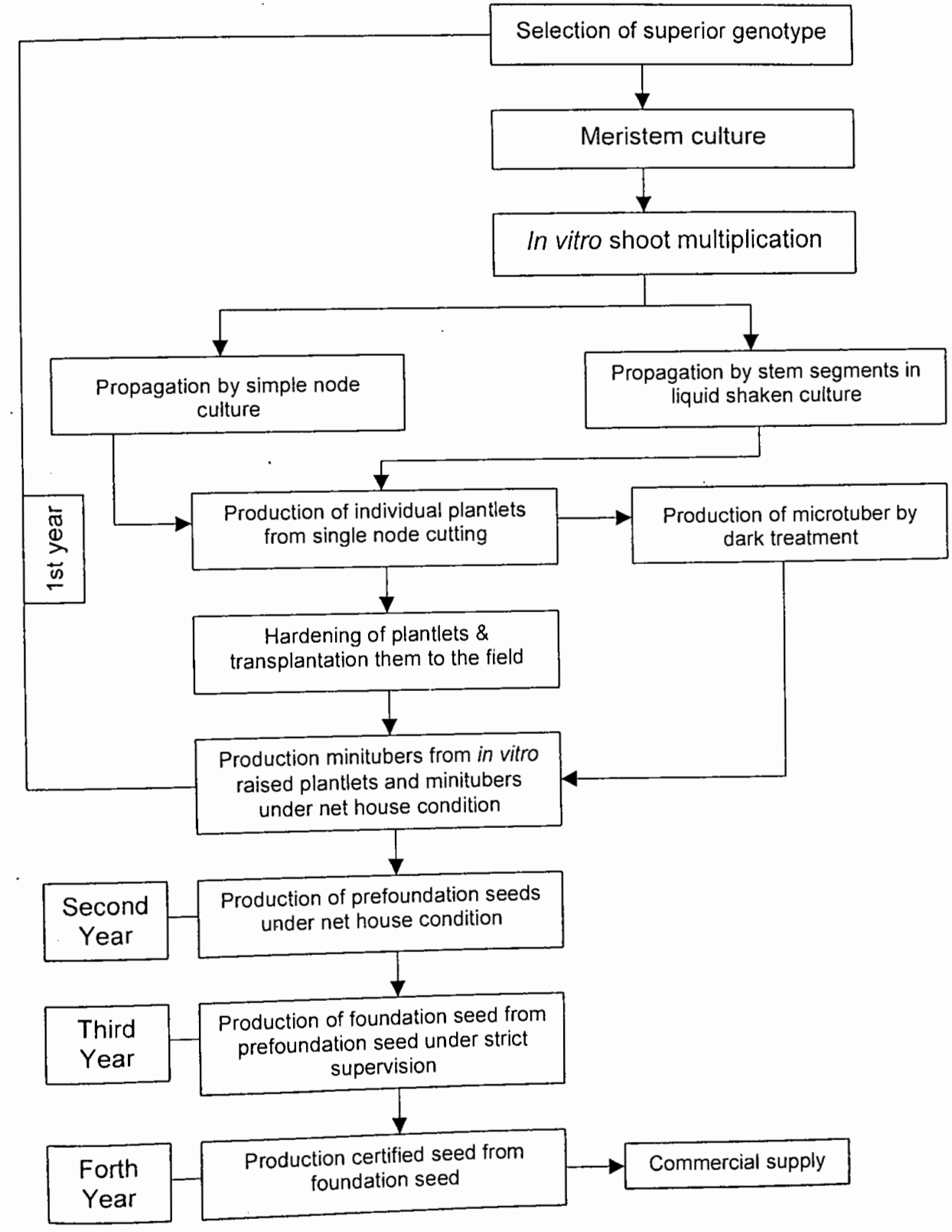
When the regenerated shoots were 2 - 8 cm in length with 5 - 10 leaves, they were rescued aseptically from the culture tubes and placed on a sterilized petridish, cut from the basal end of the shoots using sharp scalpel. Then each of the shoot base was inoculated on freshly prepared semisolid MS medium supplemented with different combinations and concentrations of growth regulators for further shoot multiplication, root induction and microtubers production.

2.2.3.6 Establishment of primary culture

Initiation of meristem culture, disease indexing, plantlet production and field transplantation were carried out according to the protocols established by Ahmed (1999) and Rahman (1998).

Over all the process of the production of disease indexed seed potatoes are shown in the following flow chart –

FLOW CHART OF VIRUS FREE POTATO SEED PRODUCTION THROUGH MERISTEM CULTURE



2.2.3.7 Micropropagation

After 4 weeks of incubation when the plantlets attained a height of 7-9 cm micropropagation was started. The plantlets were removed carefully from the test tube over a petridish using a pair of forceps. In the peridish the leaves from the stem were carefully removed and cut into single node segments.

A single node segment had undamaged axillary bud and generally it was 5-6 mm long. The isolated nodes were transferred to the culture tubes for multiplication. The node cutting was placed on the medium in such a way that it should be not pushed bellow surface of the medium. Single node cutting after incubation at 20 ± 2°C with 16h photoperiods per day grew rapidly and developed into new plantlets. These plantlets were subcultured as required after every 4 weeks.

2.2.3.8 Production of microtubers

In vitro grown shoots were also used for induction of microtubers. To induce microtubers the single node cuttings were cultured in MS medium with high concentration of BA, KIN and sucrose and were incubated at 18 ± 2°C for 8-12 weeks. The cultures were maintained at 0, 8, 24 hour photoperiods. Microtubers were harvested after required days of culture and stored at 4 °C.

2.2.4 DESIGN OF EXPERIMENTAL FIELD FOR STUDY OF MERISTEM AND MICROTUBER DERIVED PLANTS

The experiment was conducted with four varieties of potato and three sources (tuber, microshoot and microtuber) of propagules. In the layout of the experimental field complete randomized block design was followed.

Replication = 3
Total no. of beds = 36
Length of each bed = 5 m
Footpath between beds = 1 m
Footpath between boundary and beds = 0.5 m
No. of rows in each bed = 2
No. of plants in each row = 34
Total area of field = 437 m^2

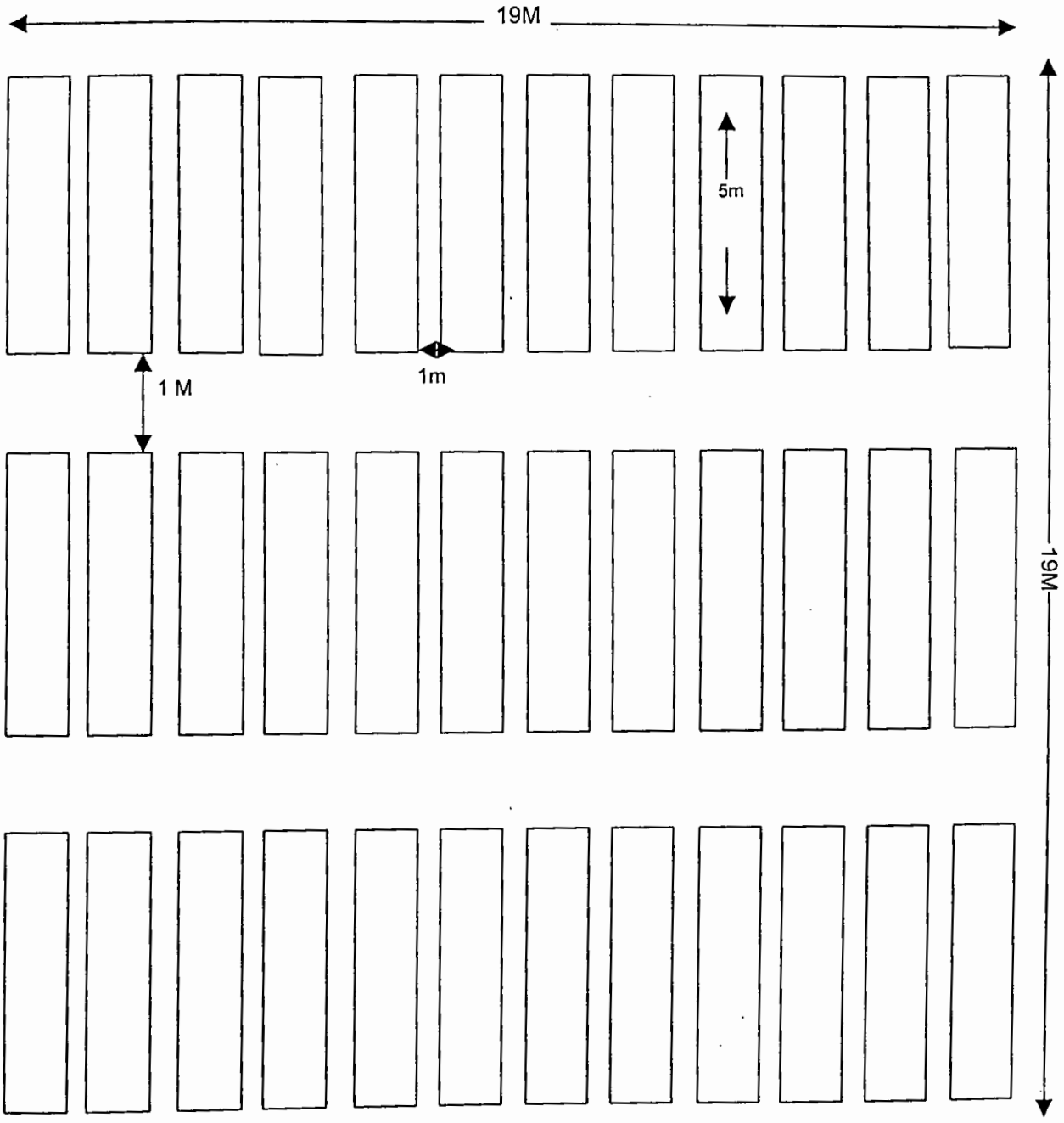


Fig. Layout of experimental field.

2.2.5 FIELD PREPARATION

The land was ploughed two to three times by a power tiller and was plained by using harrow. The weeds and debris were removed. During ploughing, the soil was pulverized with manure, fertilized, and sand was mixed with soil according to need. Before transplantation, the beds were prepared after the field was well pulverized. The length of each bed was 5 m and width was 1m. The distance between two beds was 50 cm. Before few days of transplantation the beds were treated with 1% formaldehyde solution and covered with polyethene paper for 5-6 days. After removing ploythene the beds were kept open for 3 days.

2.2.6 ACCLIMATIZATION OF *IN VITRO* GROWN PLANTLETS

When the plantlets were 6-8 cm height and developed a good root system, they were ready for transplanting into beds. Before transfer, the closures of the flasks or test tubes containing the plantlets were removed and kept in growth room for 3-5 days for acclimatization. During this period the physiological system of plant body was developed and the plantlets were more fresh and strong and were ready for transplantation.

2.2.7 TRANSPLANTATION OF PLANTLETS AND MICROTUBERS UNDER *EX VITRO* ENVIRONMENT

The plantlets were transplanted in rows keeping 12 cm space between two plants. The distance between two rows was 20 cm. Care was taken to avoid damage to the roots and to ensure good contact between roots and soil. The microtubers were planted individually also keeping 12 cm between two microtubers. The plantlets were kept in an environment with high relative humidity for the first few days following transplantation by covering the beds with polyethene paper and regular spraying of water. In order to prevent viral vectors the entire field was covered with nylon nets. Interine care was taken by periodic irrigation, weeding, mulching and spraying insecticide.

2.2.8 HARVESTING

After plantation of tissue culture raised plantlets and microtubers, potatoes were collected from the field within 90-100 days of transplantation. When haulms of potato plants started yellowing and falling on the ground, it was proper time for potato harvesting. Potato plants were cut with sickle before 7-10 days of potato harvesting. After harvesting data on tuber yield was recorded.

2.2.9 DATA RECORDING (*in vitro* culture)

Data were collected using the following parameters and the methods of data collection are given bellow –

I) Percentage of response

Percentage of explant responded was calculated using following formula;

$$\% \text{ of explant responded} = \frac{\text{Number of explant responsed}}{\text{Total number of explant cultured}} \times 100$$

II) Number of shoots / explant

Number of shoots were counted for each culture after 28 days average of shoot number was calculated and noted.

III) Shoot length

After 28 days of culture shoot length was measured in cm scales for each plantlet and average shoot length was calculated and recorded.

IV) No. of roots/shoot

Number of roots was counted for each plantlet after 28 days of subculture from 5 randomly selected cultures and the mean value were recorded.

V) Root length

Root length was measured for each plant after 28 days of subculture from 5 randomly selected plants and mean root length/plantlet was calculated.

VI) No. of microtubers/shoot

Number of microtubers were counted for each shoot after 12 weeks of culture and average microtuber number per culture was calculated.

VII) Weight of microtubers/shoot

Total weight of the induced microtubers was measured after 12 weeks of culture on the individual shoot basis from 5 randomly selected cultures and mean weight was recorded.

2.2.10 DATA RECORDING (field grown plants)

Data were collected in the field on individual plant basis from 10-12 randomly selected plants from each row in each replication for following different characters.

I) Number of leaves/plant

Total no. of leaves/plant was counted and recorded at 60 days of plantation.

II) Number of stems/plant

Total no. of stems/ plant was counted and recorded at 60 days of plantation.

III) Number of tubers/plant

The total number of tubers of individual plant was counted at the time of harvesting from 10 randomly selected plants and mean value were recorded.

IV) Weight of tubers/plant

The total weight of the tubers was measured after harvesting on the individual plant basis from 10 randomly selected plants and mean value were recorded.

2.2.11 TECHNIQUES OF ANALYSIS OF DATA

The techniques used are described under the following sub heads:

2.2.11.1 Mean

Data on individual plant were added together then divided by the total number of observations and the mean were obtained as follows:

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

Where, X = The individual reading recorded on each plant

n = Number of observations

i = 1, 2, 3, ..., n

$\sum$ = Summation

2.2.11.2 Standard error (SE)

Standard error (SE) was calculated according to following formula

$$SE = \frac{Sd}{\sqrt{N}}$$

Where,

SE = Standard Error

Sd = Standard Deviation

N = Treatment number.

2.2.11.3 Least significant difference (LSD)

Least significant different was carried out according to following formula. DMRT test was carried according to Duncan (1955) using the following formula. LSD values at 5% was calculated where the value of variance ratio for treatment effect and or cultivar effect were significant.

$$LSD = \sqrt{\frac{(\text{Error Mean Square})}{r}} \times t_{0.05} \quad \text{at (EMS) df}$$

Where,

r = Number of Replication.

Chapter - 3

OBSERVATION AND RESULTS

Chapter – 3

OBSERVATION AND RESULTS

The present investigation was carried out for the production of disease free clone through meristem culture of four varieties of potatoes (*Solanum tuberosum* L). In order to establish meristem culture and subsequent production of large number of *in vitro* plants different experiments were carried out. Details of the experiments are described under different heads.

3.1 PRIMARY ESTABLISHMENT OF MERISTEM CULTURE

3.1.1 Section of growth regulator for maximum response of meristem culture

Mesistems isolated under disecting microscope from 25-30 days old field grown plants were placed on "M" shaped filter paper bridge in culture tubes (125 × 25 mm) containing liquid MS medium supplemented with various concentrations and combinations of different plant growth regulators. Data on days to initial response and percentage of meristems responded were recorded 21 days after culture and are presented in Table 4.

Establishment of isolated meristems from 25-30 days old field grown plants were influenced by the type of cytokinines with or with out auxin and GA_3 as well as their different concentrations used. The cultured meristems commenced their initial growth by increasing in size and gradually changed to light green in colour within 5-17 days. The meristem grew further, elongated, became dark green in colour and gradually developed into minutest.

In was observed that BAP or KIN where used alone was less effective. In these cases the cultured meristems resumed new growth within 6-17 days and the percentage of explants responding ranged from 15-70%. However, of the two cytokinins used alone KIN was found to be better than BAP. (Plate 1, Fig. C). When BAP was combined with KIN or either one of these was combined with an auxin or GA_3 the range of frequency of response increased to 36-82%. The KIN with GA_3 on auxin IAA at most of the concentrations and combinations were found to be comparatively more effective than BAP with KIN or NAA (Plate 1, Figs. D & E).

In all media formulations the meristems produced shoots but few media formations produced both shoots and roots (Plate 1, Fig. F). These media were 0.1 mg/l BAP, 0.2 mg/l BAP, 0.1 mg/l KIN, 0.1 mg/l BAP + 0.1 mg/l NAA, 0.5 mg/l BAP + 0.5 mg/l NAA, 0.1 mg/l BAP + 0.1 mg/l IAA, 0.5 mg/l BAP + 0.5 mg/l IAA, 0.1 mg/l KIN + 0.1 mg/l NAA, 0.5 mg/l KIN + 0.5 mg/l NAA, 0.1 mg/l + KIN + 0.1 mg/l GA_3 and 0.5 mg/l KIN + 0.5 mg/l GA_3.

In this experiment it was observed that in media fortified with low concentrations (0.1 - 0.5 mg/l) of BAP or KIN the cultured meristems responded earlier than those of higher concentrations (1.0 - 2.0 mg/l).

Among the different treatments highest frequency (82%) of response was recorded in 0.1 KIN + 0.5 mg/l GA_3 followed by 77% in 0.1 mg/l KIN + 0.1 mg/l GA_3. But the difference between these two treatments was not significant. The lowest response (15%) was noted in 1.5 mg/l BAP.

Out of five hormonal combinations used KIN - GA_3 combination was found to be the best where more than 65% cultured meristems resumed their growth. The meristems also resumed their growth rapidly (4-10 days) in this combination.

Table 4. Effect of different concentrations and combinations of cytokinin, auxin and gibberellin in MS medium on primary response of meristems isolated from 25-30 days old field grown plants of potato cv. Diamont. Each treatment consisted of 13-15 explants. The experiment was repeated twice.

Growth regulators (mg/l)	Days to response	Percentage of explants responded	Morphogenic response	
			Shoot	Root
BAP 0.1	5-7	40	+	+
BAP 0.2	7-9	32	+	+
BAP 0.5	8-10	25	+	-
BAP 1.0	10-12	20	+	-
BAP 1.5	13-14	15	+	-
BAP 2.0	14-16	17	+	-
KIN 0.1	7-9	50	+	+
KIN 0.2	6-7	65	+	-
KIN 0.5	9-11	60	+	-
KIN 1.0	10-12	43	+	-
KIN 1.5	12-14	27	+	-
KIN 2.0	15-17	20	+	-
BAP 0.1+ KIN 0.1	5-7	45	+	-
BAP 0.2 + KIN 0.5	7-10	51	+	-
BAP 0.5+ KIN 0.1	9-12	47	+	-
BAP 0.5+ KIN 0.5	9-12	55	+	-
BAP 0.1+ NAA 0.1	10-12	36	+	+
BAP 0.5 + NAA 0.5	11-13	38	+	+
BAP 0.1+ IAA 0.1	12-14	41	+	+
BAP 0.5+ IAA 0.5	12-14	44	+	+
KIN 0.1+ NAA 0.1	9-10	52	+	-
KIN 0.5 + NAA 0.5	10-12	55	+	-
KIN 0.1+ IAA 0.1	9-10	61	+	-
KIN 0.5+ IAA 0.5	9-11	60	+	-
KIN 0.1+ GA_3 0.1	5-7	77	+	+
KIN 0.1 + GA_3 0.5	4-5	82	+	+
KIN 0.5+ GA_3 0.1	5-7	72	+	+
KIN 0.5+ GA_3 0.5	7-10	74	+	-
KIN 1.0+ GA_3 1.0	7-9	67	+	-
LSD at 5% level		9.55		

PLATE 1

PRIMARY ESTABLISHMENT OF MERISTEM CULTURE

Fig. A: Phortograph showing 25-30 days old field grown plants used for meristem isolation.

Fig. B: Isolated meristem with leaf primodia of potato cv. Diamont, 7 days after incubation on paper bridge in MS liquid medium supplemented with 0.1 mg/l BAP.

Fig. C: Isolated meristem of potato cv. Diamont, 7 days after incubation on paper bridge in MS medium with 0.2 mg/l KIN.

Fig. D: Isolated meristem of Diamont, 14 days after incubation on paper bridge in MS liquid medium with 0.1 mg/l KIN + 0.5 mg/l GA_3.

Fig. E: Isolated meristem of Diamont, 21 days after incubation on paper bridge in MS liquid medium with 0.1 mg/l KIN + 0.5 mg/l GA_3.

Fig. F: Isolated meristem of Diamont, 28 days after incubation on paper bridge in MS liquid medium with 0.1 mg/l KIN + 0.5 mg/l GA_3.

PLATE 1

3.2 SHOOT MULTIPLICATION FROM ESTABLISHED MERISTEMS

3.2.1 Selection of growth regulator for maximum shoot proliferation from established meristem

In this experiment the primary shoots developed from meristems were aseptically taken out and cut into nodal and apical bud segments. The individual shoot segments were cultured onto MS semisolid basal medium supplemented with different types of growth regulators (cytokinin, auxyn and gibberellic acid) either singly or in commination in order to find out suitable culture media for rapid shoot multiplication. Days required to shoot initiation, percentage of explants showing shoot proliferation, number of shoots per explant and length of the longest shoot were considered as parameters for evaluating this experiment. Data on these parameters from different treatments were recorded after 4 weeks of culture initiation and are presented in Table 5.

Shoot multiplication from established meristems were highly influenced by the type of cytokinins with or without auxin, GA_3 as well as their different concentrations used. The cytokinins BAP and KIN with an auxin NAA or GA_3 at most of the concentrations and combinations were found to be comparatively more effective in proliferating shoots whilst BAP and KIN singly or KIN with NAA were found to be the less effective. The cultured explants initiated shoots within 7-12 days when the medium was supplemented with cytokinin alone and the percentage of shoot proliferation ranged from 47-67%, number of shoots per explant ranged from 1.5-3.5 and the length of the longest shoot ranged from 3.1-4.5 cm. However, the performances increased significantly when BAP or KIN was combined with GA_3 or NAA in all cases. The cultured explants initiated shoots within 5-14 days when the medium was supplemented with cytokinin alone with an auxin or GA_3 and the percentage of shoot proliferation ranged from 77-85%, number of shoots ranged from 3.0-4.3 and length of longest shoot ranged from 6.1-9.6 cm. The cultured explants produced highest number of shoot per explant (4.3) in medium containing

0.5 mg/l BAP + 0.5 mg/l GA_3 (Plate 2, Fig A & B) followed by 4.1 number of shoots in medium with 0.1 mg/l BAP+0.1 mg/l GA_3 and 3.9 number of shoots in medium with 0.1 mg/l BAP + 0.1 mg/l KIN. Lowest 1.5 number of shoots per explant was obtained in medium containing 1.0 mg/l KIN.

Highest percentage of explants (85%) showed shoot proliferation was recorded in 0.5 mg/l BAP + 0.5 mg/l GA_3 followed by 81% in 0.5 mg/l BAP 1.0 mg/l IBA. Lowest 47% of shoot proliferation was observed in medium with 1.0 mg/l KIN.

Highest length of shoot 9.6 cm was recorded in medium with 0.5 mg/l BAP + 0.5 mg/l GA_3 followed by 9.3 cm in 0.1 mg/l BAP + 0.1 mg/l GA_3 and lowest length of shoot 3.1 cm was observed in medium having 0.1 mg/l BAP. From this experiment it was observed that media with three hormonal treatments *viz.* 0.5 mg/l BAP + 0.5 mg/l GA_3, 0.1 mg/l BAP +0.1 mg/l GA_3, 0.5 mg/l BAP + 1.0 mg/l IBA were proved to be favorable for multiple shoot proliferation from established meristems of potato cv. Diamont. In this three media formulation the explants started to produce shoots within 5-10 days of culture, the percentage of shoot proliferation ranged from 77-85%, number of shoots per explant ranged from 3.0-4.3 and length of the longest shoot ranged from 7.0-9.6 cm. Among these three hormonal treatments the media with 0.5 mg/l BAP +0.5 mg/l GA_3 was most effective and most preferred concentration for shoot proliferation from established meristems of potato.

Table 5: Effect different concentrations and combinations of cytokinin, auxin and gibberellin in MS medium on shoot multiplication from meristem derived shoot cultures of potato cv. Diamont. Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 explants and the treatment reapated twice.

Growth regulators (mg/l)	Days to shoot initiation	Frequency of shoot formation	No. of shoots/explant	Length of shoot (cm)
BAP 0.1	8-10	56	2.5	3.1
BAP 0.2	8-10	58	2.8	3.5
BAP 0.5	7-9	61	3.1	4.1
BAP 1.0	7-9	65	3.5	4.5
KIN 0.1	8-11	65	2.0	4.1
KIN 0.2	8-10	67	2.5	4.0
KIN 0.5	9-10	58	1.9	3.5
KIN 1.0	10-12	47	1.5	3.9
BAP 0.1+ KIN 0.1	8-10	60	3.5	6.3
BAP 0.5+ KIN 0.5	10-13	65	3.9	6.1
BAP 0.1+ IBA 0.5	7-9	66	3.1	7.1
BAP 0.5 + IBA 1.0	8-10	81	3.0	7.0
BAP 0.1+ GA_3 0.1	8-10	77	4.1	9.3
BAP 0.5+ GA_3 0.5	5-7	85	4.3	9.6
BAP 0.1+ NAA 0.5	10-12	68	3.0	9.1
BAP 0.5 + NAA 1.0	10-12	65	3.0	9.0
KIN 0.1+ NAA 0.1	12-14	61	3.1	7.5
KIN 0.5+ NAA 0.5	13-15	55	3.5	6.3
KIN 0.1+ IAA 0.1	10-12	57	3.1	8.5
KIN 0.5 + IAA 0.5	10-12	58	3.5	8.0
KIN 0.1+ GA_3 0.1	12-14	67	3.3	8.5
KIN 0.5 + GA_3 0.5	11-13	69	3.0	9.1
LSD at 5% level		7.95	0.48	1.02

3.2.2 Effect of carbon sources on multiple shoot formation

Local sugar, sucrose, glucose and fructose with three different concentrations *viz.* 20, 30 and 40 g/l for each were used for this experiment. The nodal segments raised from meristem derived *in vitro* shoots were used as explants for this experiment and they were cultured in MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l GA_3 with three different concentrations of each carbon source separately. Effect of four different carbon sources at three different concentrations on percentage of explants showing proliferation, number of shoots per explant and length of shoot were recorded after 4 weeks of culture and data are presented Table 6.

In case of local sugar it was observed that percentage of shoot proliferation ranged from 60-70%. Among the three concentrations of local sugar 30 g/l was found to be optimum concentration for maximum percentage of shoot proliferation (70%), highest number of shoot per explant (3.3) and highest length of shoot (8.0 cm) (Plate 3, Fig. A).

Percentage of explants showing proliferation in sucrose supplemented media ranged from 78-95%. Highest 95% was recorded in 30 g/l sucrose concentration and lowest 78% in 40 g/l sucrose concentration. Number of shoots per explant ranged from 4.3-4.5. Highest (4.5) number of shoots per explant was recorded in 30 g/l sucrose and lowest (4.3) number of shoots per explant was observed in 40 g/l sucrose. Length of shoot ranged from 8.5-8.6 cm. Highest length of shoot (8.6 cm) was found in 30 and 40 g/l sucrose. Lowest length (8.5 cm) was found in 20 g/l sucrose.

In case of glucose percentage of shoot proliferation ranged from 62-70%, number of shoots per explant ranged from 2.0-2.5 and length of shoot ranged 6.5-7.8 cm. Highest 70% of shoot proliferation was recorded in 40 g/l glucose, highest

(2.5) number of shoots per explant was recorded in 20 g/l glucose and the highest length of shoot 7.8 cm was recorded in 30 g/l glucose.

In fructose, it was observed that percentage of shoot proliferation ranged from 71-82%. Highest 82% shoot proliferation was recorded in 30 g/l and lowest 71% in 20 g/l fructose. Number of shoots per explant ranged from 2.0-3.0. Highest (2.3) number of shoots per explant was recorded in 30 g/l and lowest (2.0) in 20 g/l fructose (Plate 3, Fig. C). Length of shoot ranged from 4.6-5.8 cm. Highest length (5.8 cm) was found in 30 g/l and lowest (4.6 cm) in 40 g/l fructose. Among the four carbon sources studied highest (84.3%) percentage of shoot proliferation, highest (4.5) number of shoot per explant and highest (8.5 cm) length of shoot were recorded in sucrose containing media and lowest 65.0% of shoot proliferation was observerd in local sugar. Lowest number of shoots (2.1) and lowest length (5.1 cm) of shoot both were found in fructose containing media.

Among the three different concentrations studied highest 78.7% of shoot proliferation was recorded in 30 g/l and lowest 69.5% in 20 g/l concentration, highest (3.1) number of shoots per explant was recorded in 30 g/l and lowest (2.7) in 40 g/l concentration and highest length (7.5 cm) was recorded in 30 g/l and lowest (6.5 cm) in 40 g/l concentration.

From this experiment it was concluded that sucrose as a carbon source was found to be the best among the four carbon sources. Fructose was also found to be suitable. Local sugar and glucose reduced the frequency of shoot proliferation significantly.

Table 6: Effect of carbon source on shoot multiplication from nodal segments of meristem derived shoot of potato cv. Diamont in MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l GA_3. Each treatment consisted of 12-15 explants. Data were recorded after 4 weeks of culture and experiment was repeated three times.

Carbon source	Concentration (g/l)	% of explants showing proliferation	No. of shoots/explant	Length of shoot (cm)
Locol sugar	20	65	3.1	7.5
	30	70	3.3	8.0
	40	60	2.5	6.4
Sucrose	20	80	4.5	8.5
	30	95	4.7	8.6
	40	78	4.3	8.6
Glucose	20	62	2.5	7.0
	30	68	2.1	7.8
	40	70	2.0	6.5
Fructose	20	71	2.0	5.0
	30	82	2.3	5.8
	40	75	2.1	4.6
Effect of carbon source:				
Local Sugar		65.0 d	2.9 d	7.3 b
Sucrose		84.3 a	4.5 a	8.5 a
Glucose		66.6 c	2.2 c	7.1 b
Fructose		76.0 b	2.1 c	5.1 c
Effect of concentration:				
20 g/l		69.5 b	2.9 b	7.0 ab
30 g/l		78.7 a	3.1 a	7.5 a
40 g/l		70.7 b	2.7 c	6.5 b

In each colum means followed by same letters are not significantly different according to LSD at 5% level.

3.2.3 Effect of cultivar and sucrose on shoot multiplication

Four cultivars of potato *viz.* Diamont, Cardinal , Multa, Lalpakri and two concentrations of sucrose *viz.* 3% and 6% were used for this experiment. The meristem derived *in vitro* shoots were used as explant source for this experiment. Nodal segments from these *in vitro* shoots of four cultivars were cultured in MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l GA_3 with two different concentrations of sucrose. Effect of different cultivars of potato and two different concentrations of sucrose on percentage of shoot proliferation, number of shoots per explant and shoot length were recorded after 4 weeks of culture and data are presented in Table 7.

In case of media containing 3% sucrose it was observed that percentage of shoot proliferation ranged from 81-87%, number of shoots per explant ranged form 2.0-4.3 and shoot length ranged from 7.2-9.1 cm. Highest (87%) percentage of shoot proliferation was recorded in cv. Cardinal (Plate 3, Fig. E) and lowest (81%) in Lalpakri. Highest (4.3) number of shoots per explant was observed in Diamont and lowest (2.0) in Multa. Highest shoot length 9.1 cm was found in Lalpakri followed by 8.8 cm in Multa. The lowest shoot length (7.2 cm) was recorded in Cardinal.

In 6% sucrose containing media percentage of shoot proliferation ranged from 52-69%, number of shoots per explant ranged from 2.1-3.3 and shoot length ranged were 3.0-5.3 cm. Highest (69%) percentage shoot proliferation was recorded in cv. Multa (Plate 3, Fig. F) and lowest 52% in Cardinal . Highest (3.3) number of shoots per explant was found in cv. Diamont and lowest (2.1) in Lalpakri. Highest (5.3 cm) length of shoot was observed in Cardinal and lowest (3.0 cm) in Multa.

Among the two concentrations of sucrose highest (83.2%) percentage of shoot proliferation, highest (3.7) number of shoots per explant and highest (8.1 cm) length of shoot were found in 3% sucrose containg media. On the other hand all lowest parameters were found in 6% sucrose.

Among the four cultivars *viz*. Diamont, Cardinal , Multa and Lalpakri highest (76%) percentage of shoot proliferation was found in Multa and lowest (69.5%) in Diamont and Cardinal . Highest (3.8) number of shoots per explant was recorded in Diamont and lowest (2.3) in Multa. Highest (6.6 cm) length of shoot was observed in Lalpakri and lowest (5.9 cm) in Multa.

Form this experiment it was concluded that 3% sucrose containing media was found to be better than 6% sucrose for shoot multiplication in potato.

Table 7: Effect of cultivar and sucrose on % of explants developed shoot, number of shoots per explant and length of shoot. Data were recorded after 4 weeks of culture. Each treatment consisted of 12-15 explants and the experiment was repeated thrice.

Treatment		% of explants developed shoot	No. of shoots/explant	Length of shoot (cm)
Sucrose	Cultivar			
Sucrose 3%				
	Diamont	82	4.3	7.6
	Cardinal	87	4.2	7.2
	Multa	83	2.0	8.8
	Lalpakri	81	3.4	9.1
Sucrose 6%				
	Diamont	57	3.3	4.5
	Cardinal	52	3.1	5.3
	Multa	69	2.6	3.0
	Lalpakri	61	2.1	4.1
Effect of sucrose concentration				
	3%	83.2 a	3.7 a	8.1 a
	6%	59.7 b	2.7 b	4.2 b
Effect of variety				
	Diamont	69.5 b	3.8 a	6.0 c
	Cardinal	69.5 b	3.6 b	6.2 b
	Multa	76.0 a	2.3 d	5.9 c
	Lalpakri	71.0 b	2.7 c	6.6 a

In each colum means followed by same letters are not significantly different according to LSD at 5% level.

3.2.4 Effect of cultivar and number of explants per bottle on shoot multiplication

For all four cultivars *viz*. Diamont, Cardinal , Multa and Lalpakri, 6 and 8 node cuttings per bottle (12 × 5 cm) were cultured in MS medium supplemented with 0.5 mg/l BA+0.5 mg/l GA_3. Effect of 6 and 8 number of explant per bottle on number of shoot per explant, numbers of branches per shoot and shoot length were recorded 4 weeks after culture and data are presented in Table 8.

In case of 6 explants/bottle highest (4.2) number of shoots per explant was recorded for cv. Lalpakri (Plate 3, Fig. D) and lowest (3.4) in Cardinal . Highest (7.5) number of branches/shoot was observed in cv. Diamont and lowest (5.8) in Multa. Highest length (8.7 cm) of shoot was recorded in Diamont and lowest (7.5 cm) in Lalpakri.

In case of 8 explants/bottle highest number (3.5) of shoots per explant was recorded in Diamont and lowest (2.1) in Lalpakri. Highest number (4.1) of branches/shoot was found in Multa and lowest (3.1) in Cardinal . Highest (8.5 cm) shoot length was recorded in Multa and lowest (5.5 cm) in Lalpakri.

Of 6 and 8 explants/bottle highest (3.8) number of shoots/explant, and highest (8.1 cm) shoot length were found in 6 explants per bottle. But highest number of branchs/shoot was found in 8 explants/bottles. From this experiment for all four cultivars 6 explants per bottle produced more shoots and branches than 8 explants per bottle.

Table 8: Effect of cultivar and number of explants per cultur bottle on number of branches per shoots and number of shoots per bottle cultured in MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l GA_3 containing 3% sucrose. Data were recorded 4 weeks after culture.

Treatments		No. of branches per shoot	No. of shoots per explant	Length of shoot (cm)
No. of explants	Cultivar			
6 explants / bottle				
	Diamont	7.5	4.1	8.7
	Cardinal	6.3	3.4	8.6
	Multa	5.8	3.5	7.8
	Lalpakri	7.1	4.2	7.5
8 explants / bottle				
	Diamont	3.5	3.5	7.1
	Cardinal	3.1	3.0	6.7
	Multa	4.1	2.6	8.5
	Lalpakri	3.6	2.1	5.5
Effect of no. of explants / bottle				
6 explants / bottle		6.6 a	3.8 a	8.1 a
8 explants / bottle		3.5 b	2.8 b	6.9 b
Effect of variety				
	Diamont	5.5 a	3.8 a	7.9 a
	Cardinal	4.7 a	3.3 a	7.6 a
	Multa	4.9 a	3.0 a	8.1 a
	Lalpakri	5.3 a	3.1 a	6.5 b

In each colum means followed by same letters are not significantly different according to LSD at 5% level.

PLATE 2

SHOOT PROLIFERATION

Fig. A: Shoot proliferation from established meristem of Diamont cultured on MS + 0.5 mg/l BAP + 0.5 mg/l GA_3, 21 days after subculture.

Fig. B: Shoot proliferation from established meristem of Diamont cultured on MS + 0.5 mg/l BAP + 0.5 mg/l GA_3, 28 days after subculture.

Fig. C: Multiple shoot induction from nodal segments of Diamont cultured on media supplemented with MS+0.5 mg/l BAP + 0.5 mg/l GA_3 21 ddays after culture.

Fig. D: Multiple shoot induction from nodal seqments of *in vitro* grown shoot cv. Diamont cultured on media with MS+0.5 mg/l BAP + 0.5 mg/l $GA_3$21 days after culture.

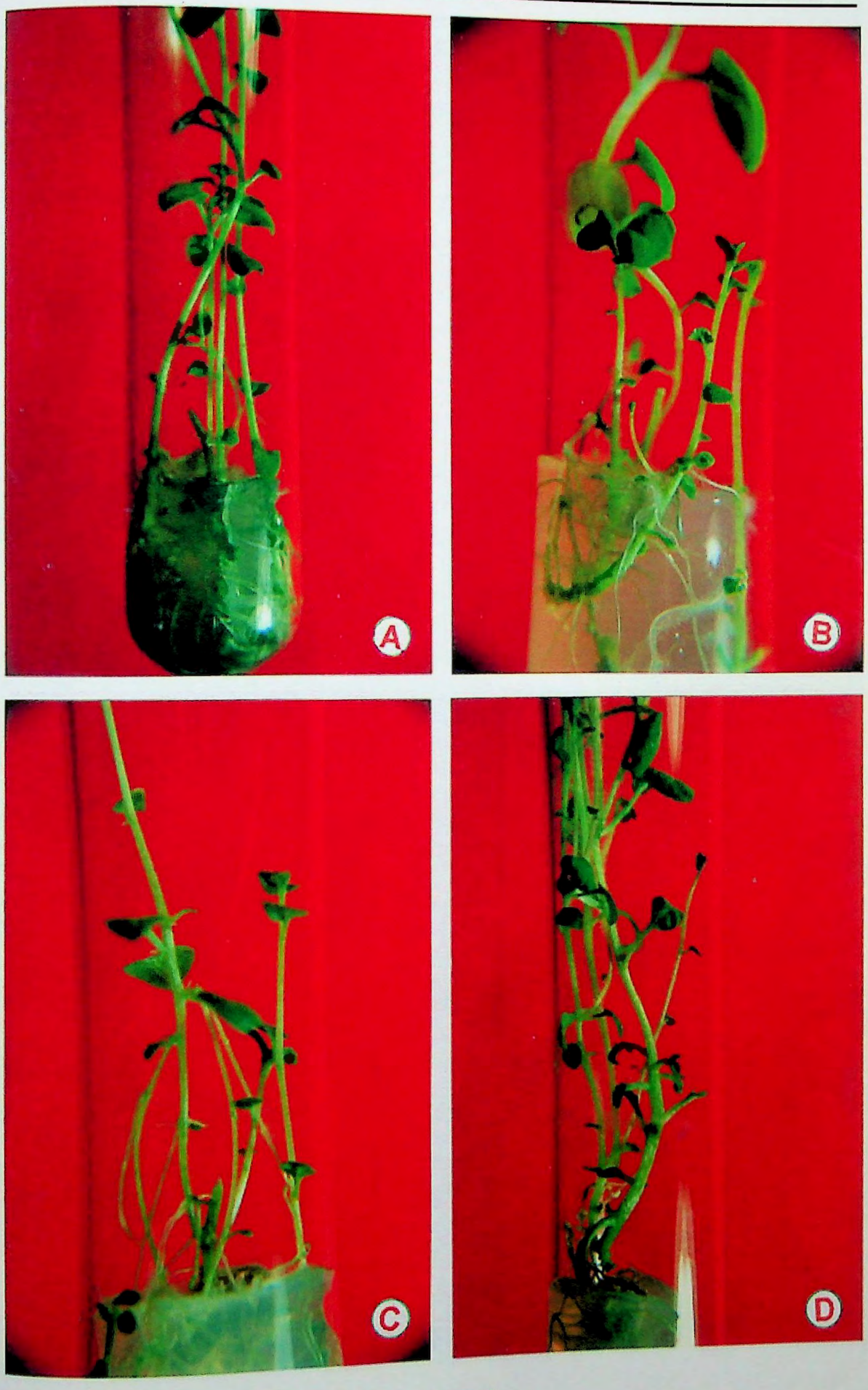


PLATE 2

PLATE 3

SHOOT PROLIFERATION

Fig. A: Shoot proliferation from nodal segments of *in vitro* derived shoot of cv. Diamont cultured on 3% local sugar containing media with 0.5 mg/l BA+0.5 mg/l GA_3, 28 days after culture.

Fig. B: Shoot proliferation from modal segments of *in vitro* derived shoots cv. Diamont cultured on 3% sucrose containing media with 0.5 mg/l BA + 0.5 mg/l GA_3, 21 days after culture.

Fig. C: Shoot proliferation from nodal segments of *in vitro* derived shoots of cv. Diamont cultured on 2% fructose containing media with 0.5 mg/l BA+0.5 mg/l GA_3, 28 days after culture.

Fig. D: Shoot proliferation from 6 nodal segments of cv. Lalpakri cultured in bottle on media with 0.5 mg/l BAP+0.5 mg/l GA_3, 28 days after culture.

Fig. E: Shoot proliferation from nodal segments of *in vitro* derived shoots of cv. Cardinal cultured on 2% sucrose containing media with 0.5 mg/l BAP+0.5 mg/l GA_3, 28 days after culture.

Fig. F: Shoot proliferation from nodal segments of *in vitro* derived shoots of cv. Multa cultured on 6% sucrose containing media with 0.5 mg/l BAP+0.5 mg/l GA_3, 28 days after culture.

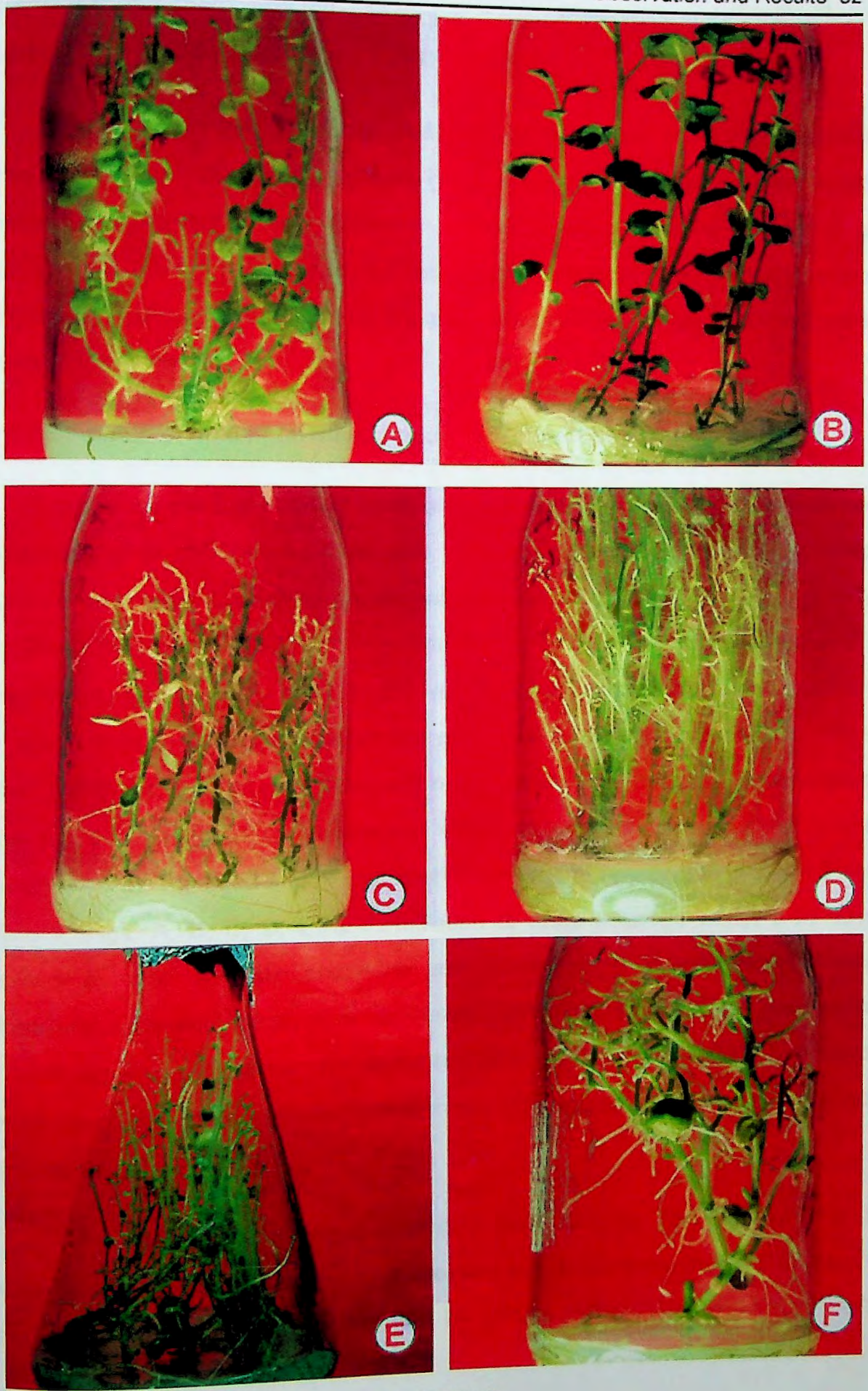


PLATE 3

3.3 ADVENTITIOUS ROOT INDUCTION

For root formation shoot segments were excised from *in vitro* grown shoot cultures. The excised shoot segments (>3 cm length) were sub-cultured on MS medium supplemented with different concentrations of auxin singly or in combination. Three different experiments were conducted to evaluate rooting efficiency of the regenerated shoots. Details of the experiments are described below under different heads.

3.3.1 Influence of auxins on adventitious root induction

In this experiment different types of growth regulators *viz.* IAA, IBA, NAA and GA_3 singly or in combination were used to induce adventitious roots. From shoot cultures developed in medium supplemented with 0.5 mg/l BAP + 0.5 mg/l GA_3, individual shoots were transferred for induction or roots. Diamont was used in this experiment. The shoots (>3 cm length) were cultured onto MS medium individually in culture tube containing media fortified with auxin singly or in combination. Effect of four different types of growth regulators on days to root initiation, percentage of root formation, number of roots/shoot, length of longest root and shoot length increment were recorded after 4 weeks of culture and are presented in Table 9 and Plate 4, Figs. A, B, C, D, E, & F. Initiation of roots occurred within 8-14 days depending upon the concentrations and combination of treatments. Media containing auxin either singly or in combination with other auxin initiated roots within 8-11 days but media without any growth regulator initiated roots within 12-14 days. Presence of GA_3 in auxin supplemented media induced roots with high shoot length increment.

Percentage of root formation ranged from 30-90%. The highest 90% of shoots induced roots was observed in medium supplemented with IBA 0.05 mg/l + GA_3 0.05 mg/l (Plate 4, Fig. C) followed by 75% in IBA 0.05 mg/l+IAA 0.05 mg/l.

Lowest 30% of root formation was recorded in NAA 0.05 mg/l+IBA 0.1 mg/l (Plate 4, Fig. E).

The average number of roots per shoot ranged from 1.2-8.5. Highest (8.5) number of roots per shoot (Plate 4, Fig. C) was recorded in IBA 0.05 mg/l + IAA 0.05 mg/l followed by 7.8 in IBA 0.05 mg/l + GA_3 0.05 mg/l (Plate 4, Fig. D). Lowest number (1.2) of roots per shoot was observed in IAA 0.05 mg/l.

Length (cm) of root ranged from 2.5-7.8 cm. Highest (7.8 cm) length of root was observed in IBA 0.05 mg/l + IAA 0.05 mg/l (Plate 4, Fig. F) followed by 7.1 cm in IBA 0.05 mg/l + GA_3 0.05 mg/l. Lowest length (2.5 cm) of root was recorded in IBA 0.05 mg/l. Degree of root induction and shoot elongation were highly influenced by the levels and types of growth regulators. The cultured shoots started to elongation within 9-14 days of culture depending upon levels and types of growth regulators. Shoot length increment ranged from 2.5-4.1 cm. Highest shoot length increment (4.1 cm) was observed in NAA 0.05 mg/l + GA_3 0.05 mg/l followed by 4.0 cm in IBA 0.05 mg/l + GA_3 0.05 mg/l. Lowest (2.5 cm) shoot length increment was recorded in IBA 0.1 mg/l (Plate 4, Fig. B).

None of the cultured shoots showed callus formation at their base in any of the formulation tested. Form the result it is evident that root formation and shoot elongation showed somewhat intricate relationship. Degree of shoot elongation also increased with the increase of rooting frequency i.e. the shoots which developed more roots they also elongated more.

Table 9: Effect of different concentrations and combinations of auxin in MS medium on adventitious rooting from *in vitro* regenerated shoot cuttings of potato cv. Diamont. Each treatment consisted of 10-12 explants and data were recorded after 4 week of culture. The experiment was repeated thrice.

Hormonal supplements (mg/l)	Days to root initiation	% of cuttings rooted	No. of roots per cutting	Length of root (cm)	Shoot length increment (cm)
00	12-14	40	2.0	2.7	2.6
IBA 0.05	10-11	49	2.1	2.5	3.2
IBA 0.1	10-11	55	2.4	2.9	2.5
IBA 0.05 + NAA 0.05	10-11	60	3.4	3.1	3.7
IBA 0.05 + IAA 0.05	8-10	75	8.5	7.8	3.9
IBA 0.05 + GA_3 0.05	8-10	90	7.8	7.1	4.0
IAA 0.05	12-14	32	1.2	2.8	2.7
IAA 0.1	12-14	35	1.4	3.0	3.0
IAA 0.05 + IBA 0.1	10-11	40	1.5	3.9	2.6
IAA 0.05 + IBA 0.05	9-11	60	5.2	5.7	3.4
IAA 0.05 + GA_3 0.05	12-14	67	7.1	6.5	3.6
NAA 0.05	12-14	35	1.5	3.0	3.1
NAA 0.1	12-14	37	1.7	3.2	3.3
NAA 0.05 + IBA 0.1	9-11	30	1.8	3.4	3.1
NAA 0.05 + IBA 0.05	9-11	58	3.1	5.4	3.3
NAA 0.05 + GA_3 0.05	12-14	45	5.4	5.7	4.1
LSD at 5% level		9.5	1.1	0.9	NS

3.3.2 Effect of sucrose on rooting

For root formation shoot segments were excised from *in vitro* grown shoot cultures. The excised shoots (>3 cm length) were cultured onto MS medium supplemented with IBA 0.05 mg/l + GA_3 0.05 mg/l and seven different levels of sucrose 10-70 g/l, gelled with agar and at pH 5.8 adjusted before autoclaving.

Presence of low and high concentrations of sucrose in the rooting medium (MS+IBA 0.05 mg/l + GA_3 0.05 mg/l) not only markedly affected the frequency of rooting, number of roots per shoot but also significantly influenced the growth and vigour of the roots. Effect of different concentrations of sucrose on days to root initiation, percentage of cuttings rooted, number of roots per shoot and length of the roots were recorded after 4 weeks of culture and the results are shown in Table 10.

Days to root initiation occurred within 8-18 days. The media with low concentration (10-40%) of sucrose induced roots rapidly (8-11 days) but when the concentration of sucrose increased (50-70%) days to root initiation was delayed.

Maximum (85%) percentage of rooting was achieved in 30 g/l sucrose concentration (Plate 5, Fig. B) followed by 80% of rooting in 20 g/l sucrose concentration. Lowest 10% of rooting was recorded in 70 g/l sucrose concentration (Plate 5, Fig. A).

The number of roots per shoot ranged from 1.2-5.1. Highest number (5.1) of roots per cutting was recorded in 40 g/l sucrose concentration followed by 4.5 number of roots in 30 g/l sucrose concentration. Lowest 1.2 number of roots per cutting was recorded in 10 g/l sucrose containing madia. Highest root length (7.7 cm) was recorded in 40 g/l sucrose concentration and the lowest root length 3.9 cm was recorded in 70 mg/l sucrose concentration.

The results reveal that media with 20 and 30 g/l sucrose were suitable for rooting where significantly higher percentage of rooting, number of roots per shoot, root length were achieved. Below and above these levels the percentage of rooting and number and the length of roots were deteriorated. The results indicate that the adventitious root formation is a energy requiring process and presence of certain level of sucrose is essential for optimum rooting.

Table 10: Effect of different concentrations of sucrose on rooting from *in vitro* regenerated shoot cuttings of potato cv. Diamont in MS medium supplemented with 0.05 mg/l IBA and 0.05 mg/l GA_3. Data were recorded after 4 weeks of culture. Each treatment consisted of 10-15 explants.

Sucrose concentration (g/l)	Days to root initiation	% of cuttings rooted	Number of roots/shoot	Length of root/shoot (cm)
10	10-11	30	1.2	4.4
20	9-11	80	3.5	8.5
30	8-10	85	4.5	6.9
40	9-11	70	5.1	7.7
50	13-16	20	4.2	5.8
60	13-16	25	4.0	5.0
70	17-18	10	2.5	3.9
LSD at 5% level		10.1	0.21	1.11

3.3.3 Genotypic response on adventitious root induction

In this experiment the excised shoot segments (>3 cm length) of four potato cultivar *viz*. Diamont, Cardinal, Multa and Lalpakri were sub-cultured on MS medium supplemented with 0.05 mg/l IBA + 0.05 mg/l GA_3. Effect of different cultivars of potato on days to root initiation, percentage of shoots induced root, number of roots per shoot and length of root were recorded after 4 weeks of culture and the result are shown in Table 11.

The results revealed that among 4 potato cultivars Diamont took very short time to root initiation and it was 8-9 days. Highest time (11-13 days) to root initiation was observed in cv. Lalpakri.

Among the four cultivars of potato it was observed that percentage of root induction ranged from 85-93%. Highest 93% root induction was observed in Cardinal (Plate 5, Fig. D) followed by 92% in Diamont. The lowest (85%) percentage of root induction was found in Lalpakri. Number of roots per shoot ranged from 3.0-3.8 and the highest (3.8) number of roots per shoot was recorded in Diamont (Plate 5, Fig.E). Lowest length of roots and number of roots per shoot were found in Cardinal. Length of root per shoot ranged from 6.2-7.1 cm and highest length (7.1 cm) of root was found in Lalpakri and lowest length of root (6.2 cm) was found in cv. Cardinal (Plate 5, Fig. F).

Table 11: Genotypic response of four potato varieties on root induction in MS medium supplemented with 0.05 mg/l IBA + 0.05 mg/l GA_3 and 30 g/l sucrose. Data were recorded after 4 weeks of culture. Each treatment consisted of 10-15 cultures and the experiment was repeated thrice.

Variety	Days to root initiation	% of shoots induced root	Number of roots/shoot	Length of root/shoot (cm)
Diamont	8-9	92 ab	3.8 a	6.8 a
Cardinal	10-12	93 a	3.0 a	6.2 a
Multa	8-10	90 ab	3.4 a	7.6 a
Lalpakri	11-13	85 b	3.1 a	7.1 a

Letters in common indicate no significant difference as determined by LSD at 5%.

PLATE 4

ROOTING OF MICROCUTTING

Fig. A: Formation of adventitious roots on shoot regenerated from nodal segments of Diamont cultured in MS Media supplemented with 0.05 mg/l IBA after 7 days of culture.

Fig. B: Formation of adventitious roots on shoot regenerated from nodal segments of Diamont cultured in MS Media supplemented with 0.05 mg/l IBA after 14 days of culture.

Fig. C: Formation of adventitious roots on shoot regenerated from shoot segments of Diamont cultured in MS + 0.05 mg/l IBA+0.05 GA_3, mg/l after 28 days of culture.

Fig. D: Formation of adventitious roots on shoot regenerated from shoot segments of Diamont cultured in MS + 0.05 mg/l IBA+0.05 mg/l GA_3, after 28 days of culture.

Fig. E: Formation of adventitious roots on shoot regenerated from shoot segments of Diamont cultured in MS + 0.05 mg/l IBA+0.1 NAA after 28 days of culture.

Fig. F: Formation of adventitious roots on shoot regenerated from shoot segments of Diamont cultured in MS + 0.05 mg/l NAA+0.05 IBA after 28 days of culture.

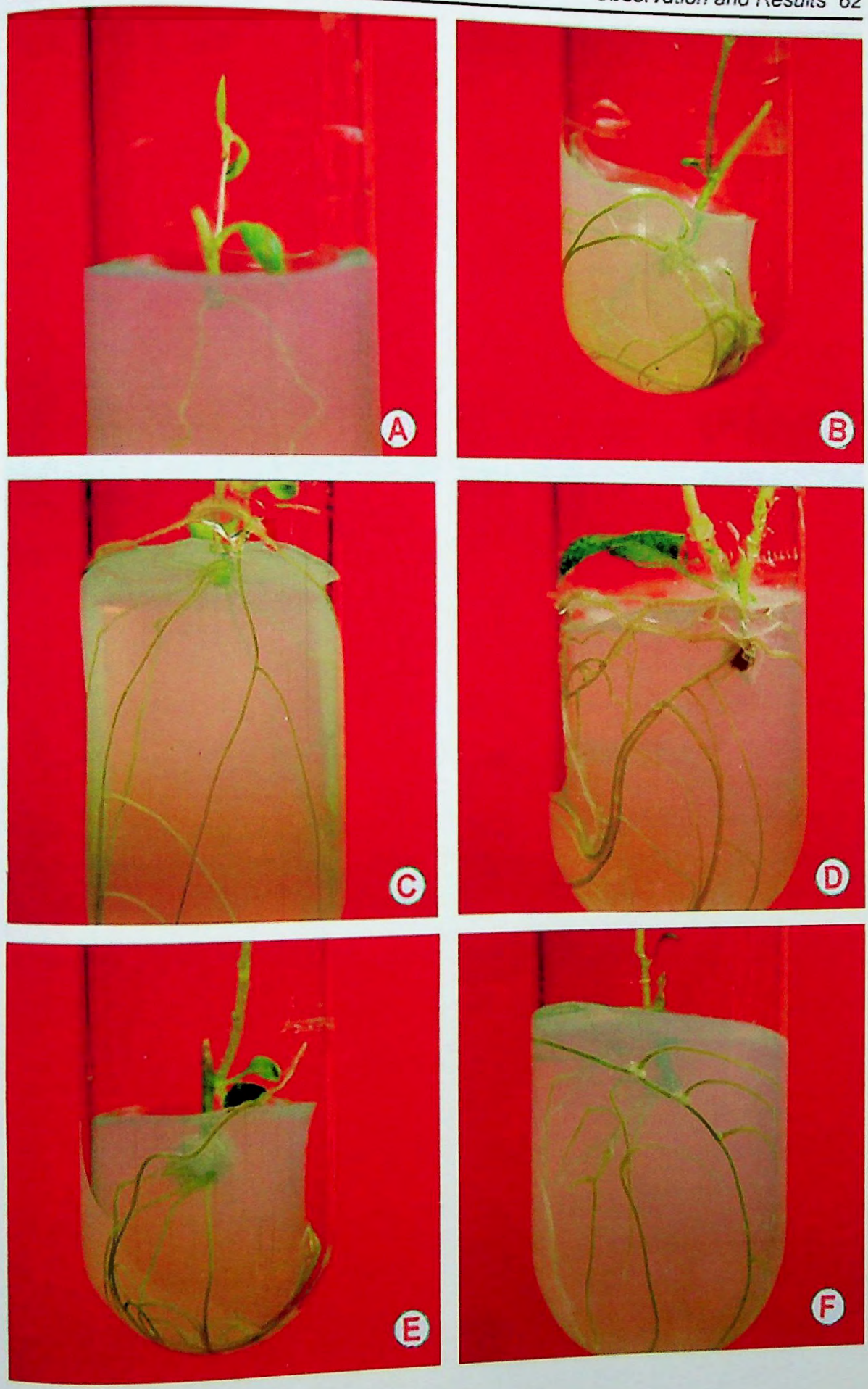


PLATE 4

PLATE 5

ROOTING OF MICROCUTTINGS

Fig. A: Formation of adventitious roots on shoot regenerated from microcutting of Diamont cultured in 50 g/l sucrose containing MS media with 0.05 mg/l IBA + 0.05 mg/l NAA, 28 days after culture.

Fig. B: Formation of adventitious roots on shoot regenerated from microcutting of Diamont cultured in 30 g/l sucrose containing MS media with 0.05 mg/l IBA + 0.05 NAA, 28 days after culture.

Fig. C: Formation of adventitious roots on shoot regenerated from microcutting of Diamont cultured MS + 0.05 mg/l IBA + 0.05 mg/l NAA, 28 days after culture.

Fig. D: Formation of adventitious roots on microcutting of cv. Cardinal cultured on MS + 0.05 mg/l IBA + 0.05 NAA, 28 days after culture.

Fig. E: Formation of adventitious roots on shoot regenerated from microcutting of Diamont cultured in containing MS media with 0.05 mg/l IBA + 0.05 NAA, 28 days after culture.

Fig. F: Formation of adventitious roots on shoot regenerated from microcutting of Cardinal cultured in containing MS media with 0.05 mg/l IBA + 0.05 NAA, 28 days after culture.

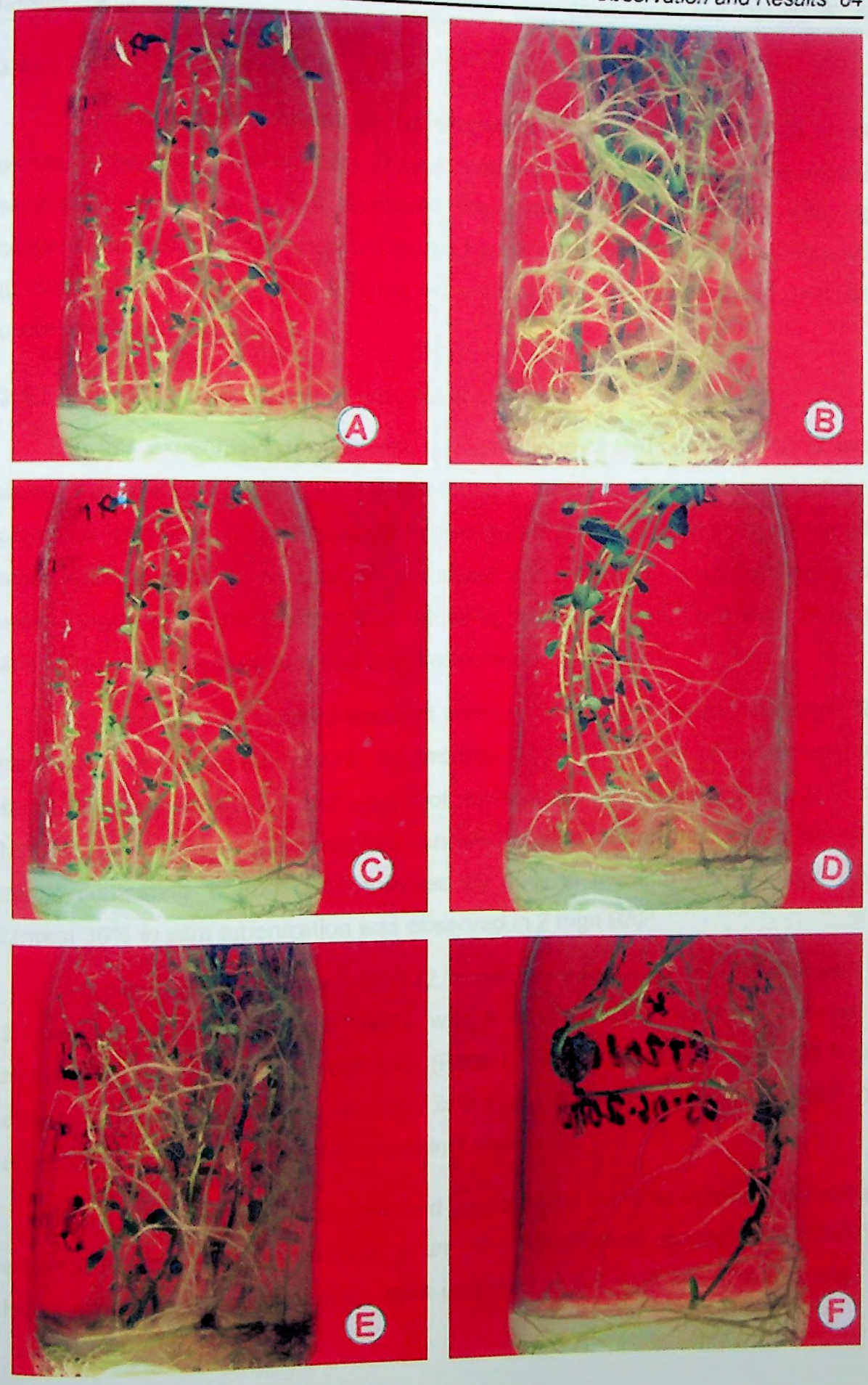


PLATE 5

3.4 *IN VITOR* TUBERIZATION

For microtuber induction nodal segments from *in vitro* multiplied shoots derived through meristem culture of four potato cultivars were used. The explants were sub-cultured on to MS semisolid basal medium supplemented with high concentration of BAP, KIN and sucrose and were incubated at different photoperiod (0, 8, 24 h) at 18±1°C. Four different experiments were conducted and described under following heads.

3.4.1 Effect BAP and KIN on *in vitro* tuberization

In this experiment six different concentrations of BAP and KIN (2-12 mg/l) were used in order to find out suitable culture media for microtuber induction in potato cv. Diamont. Weeks required to microtuber induction, percentage of *in vitro* tuberization, number of microtubers per shoot and weight of microtubers per shoot were considered as parameters for evaluating this experiment. Data were recorded after 12 weeks of sub-culture and are presented in Table 12.

In case of BAP, cultured explants induced microtubers within 7-10 days. The percentage of *in vitro* tuberization ranged from 30-57%, number of microtubers per shoot ranged from 1.3-2.7 and weight of microtubers ranged from 90.8-122.0 mg. Highest (57%) percentage of explants showed *in vitro* tubenization was obtained in media containing 12 mg/l BAP followed by 50% in medium with 10 mg/l BAP. Lowest 30% *in vitro* tubenization was observed in 2 mg/l BAP.

Highest number of microtubers (2.7) was recorded in 6 mg/l BAP followed by 2.5 number of microtubers in media with 4 mg/l BAP. Lowest 1.3 number of microtubers was found in 12 mg/l BAP (Plate 6, Fig. A). Highest weight (122.0 mg) of microtubers per shoot was recorded in 8 mg/l BAP and lowest weight (90.8 mg) of microtubers per shoot was observed in 2 mg/l BAP.

In media with KIN it was observed that cultured explants induced microtubers within 6-9 days. The percentage of *in vitro* tuberization ranged from 35-67%. Highest 67% *in vitro* tuberization was recorded in 8.0 mg/l KIN (Plate 6, Fig. B)

followed by 61% *in vitro* tuberization in 10 mg/l KIN. Lowest 35% *in vitro* tuberization was observed in 2 mg/l KIN.

Number of microtubers per shoot ranged from 2.2-3.1. The highest number of microtubers (3.1) was recorded in 8 mg/l KIN and lowest 2.2 number of microtubers was found in 12 mg/l KIN.

Weight of microtubers per shoot ranged from 112.5-218.6 mg. The highest weight (218.6 mg) of microtuber was found in 8 mg/l KIN and lowest weight (112.5 mg) of microtuber was found in 4 mg/l KIN.

From this experiment it was observed that KIN was better than BAP for microtuber induction and 8 mg/l KIN was most effective and most preferred concentration for microtuber induction.

Table 12: Effect of different concentration of BAP and KIN in MS medium with 30 g/l sucrose under continuous dark on *in vitro* tuberization of potato cv. Diamont. Each treatment consisted of 12-15 cultures. Data were recorded after 12 weeks of sub-culture and the experiment was repeated twice.

Growth regulators (mg/l)	Weeks to microtuber induction	% of *in vitro* tuberization	No. of microtubers/ shoot	Weight of microtubers/ shoot
BAP 2	8-9	30	2.1	90.8
BAP 4	8-9	32	2.5	98.3
BAP 6	7-8	45	2.7	102.4
BAP 8	7-8	47	2.3	122.0
BAP 10	8-9	50	1.8	108.6
BAP 12	9-10	57	1.3	101.9
Mean		**43.5**	**2.1**	**104.0**
KIN 2	8-9	35	2.5	129.6
KIN 4	7-8	45	2.6	112.5
KIN 6	7-8	56	2.9	184.5
KIN 8	6-7	67	3.1	218.6
KIN 10	8-9	61	2.4	201.6
KIN 12	8-9	41	2.2	187.3
Mean		**50.8**	**2.6**	**172.3**
LSD for cytokinin at 5%		6.13	0.04	10.34
LSD for concentration at 5%		4.20	0.08	15.30

3.4.2 Effect of kinetin and photoperiod *in vitro* on tuberization

This experiment (Table 13) consisted of 15 treatment combination of photoperiod and kinetin. These were three levels of photoperiod (0, 8, 24 hours) and five levels of kinetin (2, 4, 6, 8, 10 mg/l). Percentage of shoots induced microtuber, microtuber number per shoot and weight of microtuber per shoot were considered as parameters for evaluating this experiment. In this experiment only one cultivar, Diamont was used and data were recorded 12 weeks of subculture.

Among the three levels of photoperiod studied highest percentage (70.6%) of shoots induced microtubers was recorded in continuous dark. Highest number (3.51) of microtubers per shoot and highest microtuber weight (182.5 mg) were found in continuous light (24 h).

Among the five different concentrations of kinetin studied highest percentage (65%) of shoots induced microtubers was recorded in 8 mg/l KIN. Highest number (2.56) of microtubers and highest weight (151.3 mg) of microtubers were observed in 10 mg/l KIN.

From this experiment it was concluded that there was significant interaction between photoperiod and the level of kinetin. The longer the photoperiod, less effective the kinetin. In continuous light, no differences between the level of kinetin were observed for number of microtuber.

In all photoperiods increasing the level of kinetin also increased the percentage of *in vitro* tuberization, number of microtuber and weight of microtuber. Maximum increase was noticed in case of continuous dark (0 h).

Table 13: Effect of different concentrations of kinetin and photoperiod on microtuberization in potato "Diamont" after 12 weeks of sub-cultures.

Treatment		% of shoots induced microtubers	Microtuber numbers/shoot	Weight of microtubers/shoot (mg)
Photoperiods (hour)	Kinetin(mg/l)			
0 (continious dark)	2.0	45	1.72	67.3
	4.0	58	1.73	72.2
	6.0	71	1.82	98.1
	8.0	87	2.97	102.3
	10.0	92	2.21	39.6
8h light/day	2.0	32	1.03	167.8
	4.0	40	1.01	98.6
	6.0	43	1.90	103.8
	8.0	57	2.91	124.6
	10.0	48	2.10	180.5
24h light/day (continuous light)	2.0	27	3.49	118.3
	4.0	36	3.59	141.6
	6.0	40	3.50	202.6
	8.0	51	3.59	214.3
	10.0	43	3.38	235.0
Effect of photoperiod				
0		70.6	2.09	75.9
8 h		44.0	1.77	135.0
24 h		39.4	3.51	182.3
LSD at 5% level		10.21	7.01	18.12
Effect of kinetin				
2.0		34.6	2.08	117.8
4.0		44.6	2.08	104.1
6.0		51.3	2.40	134.8
8.0		65.0	3.15	147.0
10.0		61.0	2.56	151.3
LSD at 5% level		8.42	0.91	15.43

3.4.3 Effect of sucrose on *in vitro* tuberization

Four cultivars of potato *viz.* Diamont, Cardinal , Multa, Lalpakri and seven concentrations of sucrose (20-80 g/l) were used for this experiment. The meristem derived *in vitro* shoots were used as explant source for this experiment and were subcultured in MS media contained 8 mg/l KIN. Cultures were maintained under continuous light. Data were recorded after 12 weeks of subculture and are shown in Table 14.

In case of cultivar Diamont it was observed that percentage of *in vitro* tuberization ranged from 35-85%. Highest 85% was observed in media containing 60 g/l sucrose (Plate 6, Fig. D) followed by 81% in 70 g/l sucrose. Lowest 35% *in vitro* tuberization was recorded in media containg 20 g/l sucrose. Number of microtubers/shoot ranged from 1.3-4.1. Highest number (4.1) of microtuber was found in 60 g/l sucrose. Lowest (1.3) number of microtuber was observed in 20 g/l sucrose. The microtuber weight per shoot ranged from 91.5-224.5 mg. Highest microtuber weight (224.5 mg) was recorded in 60 g/l sucrose containing media (Plate 7, Fig. D) followed by 210.8 mg in 70 g/l sucrose containing media. Lowest microtuber wieight (91.5 mg) was found in 20 g/l sucrose containing media.

In cultivar Cardinal percentage of *in vitro* tuberization ranged from 36-86%. Highest percentage (86%) of *in vitro* tuberization was observed in 50 g/l sucrose containing media. Lowest 36% *in vitro* tuberization was found in 20 g/l sucrose. Highest number (4.2) of microtaber per shoot was recorded in 50 g/l sucrose containing media. Lowest 1.2 number of microtuber was found in 20 and 70 g/l sucrose (Plate 7, Fig. C). Highest microtuber weight (221.5 mg) was found in 40 g/l sucrose containing media.

In case of Multa it was observed that percentage of shoots induced microtubers ranged from 31-84%. Highest percentage (84%) of shoots induced

microtuber in 60 g/l sucrose containing media, followed by 79% in 70 g/l sucrose containing media. Lowest 31% shoots induced microtubers in medea containg 80 g/l sucrose. Highest number (4.0) of microtubers was found in 50 g/l sucrose containing media. Lowest mumber (1.4) of microtuber was recorded in media supplied with 20 g/l sucrose. Highest microtuber weight (221.8 mg) was recorded in 60 g/l sucrose supplemented media and lowest 87.5 mg microtuber per shoot was found in 20 g/l sucrose, containing media (Plate 6, Fig. C).

In cultivar Lalpakri percentage of *in vitro* tuberization ranged from 30-81%. Highest percentage (81%) of *in vitro* tuberization was observed in 60 g/l sucrose containing media. Lowest 30% *in vitro* tuberization was found in 80 g/l sucrose supplemented. Highest number (3.5) of microtaber per shoot was recorded in 60 g/l sucrose containing media. Lowest 1.0 number of microtuber was found in 20 g/l sucrose. Highest microtuber weight (147.6 mg) was found in 60 g/l sucrose containing media.

Among the four cultivars *viz.* Diamont, Cardinal, Multa and Lalpakri highest percentage (82.2%) of shoots induced microtubers was found in 60g/l sucrose containing media. Lowest 38% *in vitro* tuberization was found in 20 g/l sucrose added media. Highest number (3.8) of microtuber per shoot was found in 60 g/l sucrose containing media. Highest microtuber weight (203.6 mg) was found also in 60 g/l sucrose supplemented media.

From this experiment it was concluded that percentage of *in vitro* tuberization and weight of microtuber were increased when the concentration of sucrose was increased. In media containing 20-50 g/l sucrose more or less similar response in respect of microtuber formation was observed. The media containing 60 g/l sucrose was found to be the best among seven sucrose concentrations.

Table 14: Effect of different concatenations of sucrose and cultivar on *in vitro* tuberization in potato. MS media contained 8 mg/l KIN and cultures were maintained under continuous light. Data were recorded after 12 weeks of subculture. Each treatment consisted of 10-12 cultures and the experiment was repeated twice.

Sucrose concentration (g/l)	Diamont	Cardinal	Multa	Lalpakri	Mean	LSD at 5% level
			% of shoots induced microtuber			
20	35	36	39	42	38	
30	51	49	45	47	47	
40	65	67	61	60	63.2	
50	78	86	75	74	78.2	8.3
60	85	79	84	81	82.2	
70	81	83	79	71	78.5	
80	53	60	31	30	43.5	
Mean	64	65.7	59.1	57.8		
			Number of microtubers/shoot			
20	1.3	1.2	1.4	1.0	1.2	
30	2.7	2.5	2.8	2.0	2.6	
40	3.0	2.9	3.1	2.6	2.9	
50	3.7	3.8	4.0	3.1	3.6	1.0
60	4.1	4.2	3.6	3.5	3.8	
70	3.1	1.2	3.2	2.8	2.5	
80	1.9	2.8	1.8	1.2	1.9	
Mean	2.8	2.6	2.8	2.3		
			Microtuber weight (mg)/shoot			
20	91.5	90.3	87.5	57.1	81.6	
30	98.2	95.6	91.6	78.7	91.0	
40	132.4	121.5	130.3	91.3	118.8	
50	181.5	180.6	178.6	102.5	161.0	16.2
60	224.5	220.6	221.8	147.6	203.6	
70	210.8	209.7	208.6	135.3	191.1	
80	102.4	101.5	98.5	76.0	94.6	
Mean	148.7	145.6	145.2	98.3		

3.4.4 Effect of kiniten and cultivar on *in vitro* tuberization

Four cultivars of potato *viz.* Diamont, Cardinal, Multa, Lalpakri and three concentrations of kinetin (6, 8, 10 mg/l) were used for this experiment. The meristem derived *in vitro* shoot were used as explant source for this experiment. Cultures were maintained under continuous light. Data were recorded after 12 weeks of subculture and are shown in Table 15.

In case of cultivar Diamont it was observed that percentage of *in vitro* tuberization ranged from 67-91%. Highest 91% was observed in media containing 8.0 mg/l KIN followed by 76% in 6.0 mg/l KIN. Lowest 67% *in vitro* tuberization was recorded in media supplemented 10 mg/l KIN. Number of microtuber/shoot ranged from 3.60-3.72. Highest number (3.72) of microtuber was found in 6.0 mg/l KIN. Lowest (3.6) number of microtuber was observed in 10 mg/l KIN. The microtuber weight per shoot ranged from 187.6-230.7 mg. Highest microtuber weight (230.7 mg) was recorded in 8 mg/l KIN containing media. Lowest microtuber wieight (187.6 mg) was found in 6 mg/l KIN fortified media.

In cultivar Cardinal highest percentage (87%) of *in vitro* tuberization was observed in 8 mg/l KIN containing media. Lowest 61% *in vitro* tuberization was found in 10 mg/l KIN. Highest number (3.99) of microtuber per shoot was recorded in 8 mg/l KIN containing media. Lowest 3.82 number of microtuber was found in 6 mg/l KIN. Highest microtuber weight (232.6 mg) was found in 8 mg/l KIN containing media.

In case of Multa it was observed that highest percentage (89%) of shoots induced microtubers in 8 mg/l KIN containing media. Lowest 59% of shoots induced microtubers in medea containing 10 mg/l KIN. Highest number (3.76) of microtubers was found in 8 mg/l KIN fortified media. Lowest mumber (3.63) of microtuber was recorded in media with 10 mg/l KIN. Highest microtuber weight (221.3 mg) was

recorded in 8 mg/l KIN added media and lowest weight (177.5 mg) of microtuber per shoot was found in 6 mg/l KIN supplemented media.

In cultivar Lalpakri highest percentage (90%) of *in vitro* tuberization was observed in media with 8 mg/l KIN. Lowest 78% *in vitro* tuberization was found in 6 mg/l KIN. Highest number (3.38) of microtaber per shoot was recorded in 6 mg/l KIN containing media. Lowest 3.20 number of microtuber was found in 8 mg/l KIN. Highest microtuber weight (212.6 mg) was found in 8 mg/l KIN containing media.

Among the four cultivars *viz.* Diamont, Cardinal, Multa and Lalpakri highest percentage (85.0%) of shoots induced microtubers was recorded in Lalpakri. Lowest 71% *in vitro* tuberization was found in cv. Multa. Highest number (3.89) of microtuber per shoot was found in Cardinal. Highest microtuber weight (204.8 mg) was found also in Cardinal.

Among the three concentrations of kinetin (6, 8, 10 mg/l) highest percentage (89.2%) of shoots induced microtubers was observed in media supplemented with 8 mg/l KIN. Lowest 68.5% of shoots induced microtubers was found in 10 mg/l KIN containing media. Highest number (3.65) of microtuber was found in 6 mg/l KIN added media. Highest weight (224.3 mg) of microtuber was found in 8 mg/l KIN fortified media.

From this experiment it was concluded that 8 mg/l KIN was found to be most effective for *in vitro* tuberization in potato.

Table 15: Effect of different concentration kinetin and cuiltivar on % of shoots induced microtuber number of microtuber and weight of microtuber after 12 weeks of subcultue and under continuous light.

Treatment		% of shoots induced microtuber	Number of microtubers /shoot	Weight of microtuber/shoot (mg)
Cultivar	Kinetin(mg/l)			
Diamont	6.0	76	3.72	187.6
	8.0	91	3.61	230.7
	10.0	68	3.60	191.3
Cardinal	6.0	78	3.82	188.5
	8.0	87	3.99	232.6
	10.0	61	3.88	193.5
Multa	6.0	65	3.65	177.5
	8.0	89	3.76	221.3
	10.0	59	3.63	197.0
Lalpakri	6.0	78	3.38	167.3
	8.0	90	3.20	212.6
	10.0	87	3.21	176.5
Effect of variety				
Diamont		78.0	3.64	203.2
Cardinal		75.3	3.89	204.8
Multa		71	3.68	198.6
Lalpakri		85	3.26	186.1
LSD at 5% level		9.0	NS	20.2
Effect of conciliation				
6.0 mg/l		77.2	3.65	180.2
8.0 mg/l		89.2	3.64	224.3
10.0 mg/l		68.5	3.58	189.5
LSD at 5% level		10.0	NS	22.2

PLATE 6

MICROTUBER INDUCTION

Fig. A: Microtuber induction from meristem derived *in vitro* shoots of Diamont cultured in 12 mg/l BAP under continuous dark, 12 weeks after subculture.

Fig. B: Microtuber induction from meristem derived *in vitro* shoots of Diamont cultured in 12 mg/l KIN under continuous dark, 12 weeks after subculture.

Fig. C: Microtuber induction from meristem derived *in vitro* shoots of Multa cultured in 20 g/l sucrose containing media with 8 mg/l KIN.

Fig. D: Microtuber induction from meristem derived *in vitro* shoots of Diamont cultured in 60 g/l sucrose containing media under continuous light.

PLATE 6

PLATE 7

MICROTUBER INDUCTION

Fig. A: Microtuber induction from meristem derived *in vitro* shoots of Diamont cultured in 2 mg/l KIN under continuous dark.

Fig. B: Microtuber induction from meristem derived *in vitro* shoots of Diamont cultured in 4 mg/l KIN under 8 hour photoperiods.

Fig. C: Microtuber induction from meristem derived *in vitro* shoots of Cardinal cultured in 20 g/l sucrose containing media with 8 mg/l KIN, 12 weeks of subculture.

Fig. D: Microtuber induction from meristem derived *in vitro* shoots of Diamont cultured in 70 g/l sucrose containing media with 8 mg/l KIN, 12 weeks of subculture.

PLATE 7

3.5 DORMANCY OF MICROTUBER

3.5.1 Dormant period of microtuber

In this experiment three concentrations (6, 8, 10 mg/l) of kinetin and microtubers of four cultivars of potato were used in order to measure dormant period of the induced microtubers. The common method of mesuring dormancy in the potato is from harvest to sprouting.

There was little effect of cultivar and level of kinetin on the length of the dormant period as measured from harvest to sprouting. Measuring dormany from tuber initiation to sprouting showed a pronounced effect of kinetin (Table 16). The kinetin level of 8 mg/l shortened the dormant period for all cultivars. Storage temperature of 20±1°C was used in all these experiments.

3.5.2 Rate of emergence of microtuber

The time of emergence of planted and sprouting microtubers averaged from 10 to 17 days (Table 17). At planting Lalpakri had a more uniform and longer sporut (1 to 2 mm), whereas the sprout length of other cultivars was generally less than 1 mm. This was one of the reasons why Cardinal and Lalpakri had the earliest emergence with small variation in time of emergence (Table 17). For earlier emergence and uniform emergence, microtubers with sprout length of about 2 mm is recommended.

3.5.3 Rate of growth of microtuber

Ten node stage is an appropriate size of plant for transprtanting in to the field. Most of the plants observed produced compound leaves after the seventh node. The rate of microtuber plant growth from emergence to ten node stage took 18 to 23 days. A shorter period (+18 days) was required for Diamont, Lalpakri Cardinal, and a longer period (+23 days) for Multa (Table 18). At this stage, the plant had reached a height of 14 to 16 cm (Table 18).

Table 16: The dormant period of kinetin-induced microtubers of 4 cultivers of potatos grown under continuos light.

Treatment		Microtuber initiation to harvest (wks)	Harvest to sprouting (wks)	Microtuber initiating to sprouting (wks)
Cultivar	Kinetin (mg/l)			
Diamont	6.0	8	8	16
	8.0	8	8	16
	10.0	6	8	14
Cardinal	6.0	12	6	18
	8.0	12	5	17
	10.0	8	6	14
Multa	6.0	8	7	15
	8.0	8	7	17
	10.0	8	6	14
Lalpakri	6.0	8	8	16
	8.0	8	7	15
	10.0	7	9	16

Table 17: Date of sprout emergence of planted and sprouting microtubers of four cultivars of potato.

Cultivar	Days to Emergence	
	Average	100%
Diamont	14	26
Cardinal	10	22
Multa	16	28
Lalpakri	17	20

Table 18: Duration of growth period of micropropagated plants from emergence to transplanting stage (10 node long shoot) and height at transplanting stage.

Cultivar	Duration in days	Height (cm)
Diamont	18	14.3
Cardinal	19	14.8
Multa	23	13.8
Lalpakri	18	15.8

3.6 PREPARATION PERIOD FOR FIELD TRANSPLANTING

In potato meristem culture, micropropagation, the preparation period can be subdivided into several substages as follows:

Substage I: The breakage of dormancy.

Substage II: The pregrowth period: this includes the rooting of microshoots and the emergence of shoots form microtubers.

Substage III: Development and early shoot growth: the early growth of plants before hardening.

Substage IV: The hardening for field condition.

The required time needed to produce transplantable micropropagated plants starting from the meristem culture stage is summarized in Table 19. A longer time was required to produce micropropagated plants from microtubers (18-32 weeks) than from microshoots (10 weeks). The principal difference is due to the extensive period needed to produce microtubers in culture and the additival time needed to break the dormancy of microtubers. There was no difference in time requirements between microshoots and microtubers from the substage II, III and IV (Table 19). Even through microtubers have a longer period from the production of transplantable plants, microtubers have the advantage of being more readily stored and shipped than microcuttings.

Table 19: The total time required to obtain a transplantable micropropagated plants (10 node plant) from a microshoot or a microtuber.

Stage or substage	Microshoot	Microtuber
Stage 1. Meristem culture	2 wks	–
Stage 2. Multiplication	4 wks	8-12 wks BAP, KIN-dark 12-16 wks KIN-light
Stage 3. Preparation of field planting	–	–
Substag I. Dormancy	–	6-10 wks for sprouting of microtubers
Substag II. Pregrowth period	1 wks (rooting)	1-2 wks (emergence)
Substag III. Development and growth period	2 wks	2 wks
Substag IV. Hardening	1 wks	1 wks
	10 wks	**18 - 32 wks**

3.7 COMPARATIVE FIELD PERFORMANCE OF POTATO PLANTS DERIVED FROM TUBERS, MICROSHOOTS AND MICROTUBERS

Plantlets were raised from meristems of Diamont, Cardinal, Multa and Lalpakri using the optimum culture condition. Microtubers were also induced on microshoots of all these four varieties.

The field was prepared with garden soil, compost and sand (1:1:1) and moistened uniformaly. Plantlets were removed from the water and planted individully. After breaking dormancy the microtubers were also sown at the same time, taking special care not to damage the propagule sources. For the first 1-2 weeks of transfer, the plants were kept covered with polythene paper for providing the conditions of high humidity. The polythene covers were removed periodically and progressively whenever leaves appear water soaked on polyethene cover look foggy. After resuming the now growth on soil and having sufficient extension of growth and leaf development the polythene covers were removed completely. The plants were watered regularly and upper layer of soil was mixed and mulched occasionally. Data were collected from randomly selected plants. The data were analyzed for the justified assessment interpretation of the results and are shown in Table 20-22 (Plate 8).

3.7.1 Morphological characters and yield performance of tubers, microshoot and microtuber derived plants.

Plantlets were raised from meristems derived plantlets of four potato cultivars *viz*. Diamont, Cardinal , Muta and Lalpakri. Microtubers were also induced from all these varieties. Four growth parameters such as number of stems per plant, number of leaves per plant, number of tubers per plant and tuber weight per plant were recorded to evaluate performance of three propagule sources (tuber, microshoot, microtuber). Morphological data were recorded at 60 days and yield characters were recorded at harvesting (90 days) and are shown in Table 20, Plate 8.

For all four cultivars of potato *viz*. Diamont, Cardinal, Multa and Lalpakri, plants from seed tubers had a greater number (4.1-5.6) of stem than plants from

microcultures (microshoot, microtuber). Between microshoot and microtuber there was no difference in number of stems per plant. In plants from microtuber number of stems per plant ranged from 1.0-1.3 (Plate 8, Fig. E).

Among the three propagule sources for all cultivars greater number (182.6-240.3) of leaves per plant was recorded in plants produced form microculture (microshoot, microtuber). Average 216 numbers of leaves were found in plants from microshoots.

At harvesting period, plants from microculture (microshoot, microtuber) had a greater number of tubers per plant than tuber produced plants (Table 20). This difference was significant for all the cultivars. For all four cultivars highest number of tubers per plant was fond in the plants from microshoots and the number ranged from 21.8-38.6. Highest 38.6 number of tubers per plant was recorded in microshoots of Multa.

Maximum tuber weight per plant was found in tuber-produced plants among plants produced by the three propagation methods for all cultivars. Highest tuber weight (479.3 g) was found in tuber-produced plants (Cardinal). Of microshoot and microtuber highest tuber weight (232.3 g) per plant was noted in the plants from microshoots of cv. Diamont, (Plate 8, Fig. D).

Table 20: The yield of four potato cultivars gown in the field under net house condition produced from three propagation sources.

Propagule source (treatment)	Number of stems/plant	Number of leaves/plant	Number of tubers/plant	Total tuber weight/plant (g)
Diamont				
Tuber	4.5	138.5	11.6	469.5
Microshoot	1.2	207.6	21.8	232.3
Microtuber	1.0	212.8	18.3	190.2
Cardinal				
Tuber	5.6	129.9	12.5	479.3
Microshoot	1.1	240.3	23.6	209.5
Microtuber	1.3	189.5	21.1	200.1
Multa				
Tuber	4.1	172.5	13.8	393.5
Microshoot	1.0	212.6	38.6	231.6
Microtuber	1.1	192.5	23.3	192.8
Lalpakri				
Tuber	4.6	152.3	10.6	387.6
Microshoot	1.3	203.5	31.5	190.3
Microtuber	1.2	182.6	23.6	172.6
Effect of cultivar				
Diamont	2.2	186.3	17.2	297.3
Cardinal	2.6	186.5	19.0	296.3
Multa	2.0	192.5	25.2	272.6
Lalpakri	2.3	179.4	21.9	250.1
LSD at 5% level	**NS**	19.2	**2.0**	**25.4**
Effect of propogule sources				
Tuber	4.7	148.3	12.1	432.4
Microshoot	1.1	216.0	28.8	215.9
Microtuber	1.1	194.3	21.5	188.9
LSD at 5% level	1.0	**20.0**	**2.3**	**27.4**

3.7.2 Viral and bacterial infection of potato cultivars produced from tuber, microshoots and microtubers

Potato crops sulfers from various viral, bacterial and fungal discases. Potato plants are contaminated with virus such as PVS, PVM, PVY, PVX and PLRV. Yield obtained from infected plants are very low. To evaluate the disease reaction three propagation sources-tuber, microshoot and microtuber of four cultivers of potato *viz.* Diamont, Cardinal, Multa and Lalpaki were used.

Disease reaction was visualized throughout the growing period. Data were recorded at 20, 40 and 60 days after plantation and are presented in Table 21.

Among the three propagule sources for all cultivars only tuber produced plants were contaminated by virus at 20 days after plantation. At 60 days after plantation few (2.1-4.7%) plants from microculture (microshoot, microtuber) were contaminated by virus for all cultivars. So, throughout the growing period (20, 40, 60 days after plantation) greater frequency of viral infection was observed in tuber produced plants than the plants from microculture (microshoot, microtuber) and of two microcultures (microshoot, microtuber) greater frequency (4.7%) of viral infection was observed in plants obtained from microshoots at 60 days after plantation.

There are many bacterial diseases occurred in potato plants such as brown rot, black leg, bacterial ring rot etc. To identify the bacterial diseases in potato plants produced from three propagule sources data were recorded at 20, 40 and 60 days after plantation.

At 20 days after plantation among the three propagation sources in all cultivars only tuber produced plants were contaminated by bacteria and the frequency of bacterial infection ranged from 1.0 - 2.6. No bacterial infection was

observed in the plants from microculture (microshoot, microtuber) at 20 days after plantation.

At 40 days after plantation very low frequency of bacterial infection was recorded in the plants from microculture and the frequency of infection ranged from 1.0-1.6.

On 60 days, greater frequency of bacterial infection was observed in tuber produced plants than the plants from microculture (microshoot, microtuber). In tuber produced plants highest percentage (5.1%) was found in cv. Diamont and lowest 3.1% bacterial infection was noted in cv. Lalpakri. Of microshoot and microtuber derived plant maximum 2.7% bacterial infection was observed in the plants from microshoots of cv. Diamont among the four cultivars.

Table 21: Viral and bacterial disease reaction of tuber, microshoot and microtuber derived derived plants of four potato cultivars grown under net house condition.

Propagule source (treatment)	Viral infection (%) Days after plantation			Bacterial infection (%) Days after plantation		
	20 days	40 days	60 days	20 days	40 days	60 days
Diamont						
Tuber	3.0	6.5	9.6	2.6	3.5	5.1
Microshoot	0	2.5	3.1	0	1.5	2.7
Microtuber	0	2.0	2.5	0	1.0	2.5
Cardinal						
Tuber	2.6	5.5	8.7	1.5	2.5	3.9
Microshoot	0	2.9	4.0	0	1.6	2.5
Microtuber	0	2.1	3.0	0	1.2	1.9
Multa						
Tuber	3.5	7.1	10.5	1.0	2.1	4.5
Microshoot	1.0	3.5	4.7	0	1.5	2.0
Microtuber	0	1.1	2.5	0	1.0	1.5
Lalpakri						
Tuber	2.7	6.1	7.6	1.3	2.3	3.1
Microshoot	0	2.1	3.0	0	1.0	1.5
Microtuber	0	0	2.1	0	1.0	1.0

3.7.3 Foliar late blight response of potato cultivars produced from tuber, microshoots and microtubers

Potato caused by *Phytophthora infestans* remains a major disease that can rapidly destroy a crop in the field or in storage if not controlled properly. The use of meristem culture technique are generally adopted to produce disease free plants. To evaluate the discase reaction three propagation sources tuber, microshoot and microtuber of potato cultivars *viz*. Diamont, Cardinal, Multa and Lalpakri were used to study disease reaction and the reaction was visualized throughout the growing period. Data were recorded at 20, 30, 40, 50, 60 and 70 days after plantation and are presented in Table 22.

On 20 days after plantation among the three propagation sources in all cultivars frequency of foliar damage of tuber produced plants was higher than plants obtained from microcultures (microshoot, microtuber) and the frequency of foliar damage ranged from 7.6-9.1%. Between microshoot and microtuber maximum 0.5% foliar damage was observed in the plants obtained from microshoots of cv. Cardinal at 20 days after plantation.

Throughout the growing period it was observed that frequency of late blight foliar damage of tuber produced plants for all cultivars was higher than the plants obtained from microcultures.

At 70 days after plantation frequency of late blight foliar damage of tuber produced plants for all cultivars ranged from 9.2-11.9. Highest 11.9% foliar damage was observed in tuber produced plants of cv. Cardinal. Between microshoot and microtuber produced plants maximum 5.5% foliar damage was observed in the plants obtained from microshoots of cv. Multa. Lowest 1.2% foliar damage was observed in microtuber produced plants of cv. Lalpakri.

Table 22: Foliar late blight response of four cultivars of potato grown under net house condition and produced from three propagation sources. Propagule sources were transplanted on December 15.

Propagule source (treatment)	Late blight foliar damage (%) days after plantation					
	20 days	30 days	40 days	50 days	60 days	70 days
Diamont						
Tuber	8.5	9.1	9.2	9.3	9.7	9.8
Microshoot	0.2	0.5	3.3	3.5	3.7	4.0
Microtuber	0	0.2	1.2	1.7	2.0	2.1
Cardinal						
Tuber	9.1	10.0	11.5	11.7	11.8	11.9
Microshoot	0.5	1.3	4.7	4.9	5.1	5.4
Microtuber	0.2	0.5	2.3	3.0	3.1	3.5
Multa						
Tuber	8.7	9.3	9.8	10.3	10.3	10.5
Microshoot	0.3	1.3	4.5	5.1	5.3	5.5
Microtuber	0.3	1.0	1.4	2.0	2.1	2.2
Lalpakri						
Tuber	7.6	8.5	8.7	9.0	9.1	9.2
Microshoot	0	0.5	1.2	1.8	2.0	2.1
Microtuber	0	0.8	0.9	1.0	1.0	1.2

PLATE 8

FIELD PERFORMANCE OF *IN VITRO* RAISED PLANTS UNDER NET HOUSE CONDITION

Fig. A: Plants in net house, developed from meristem derived plants, 45-days after plantation.

Fig. B: Plants in net house, developed from meristem derived plants, 70-days after plantation.

Fig. C: Tubers developed from neristem derived plant of Cardinal, 90-day after plantation under net house condition.

Fig. D: Tubers developed from neristem derived plant of Diamont, 90-day after plantation under net house condition.

Fig. E: Tubers developed from neristem derived plant of Lalpakri, 90-day after plantation under net house condition.

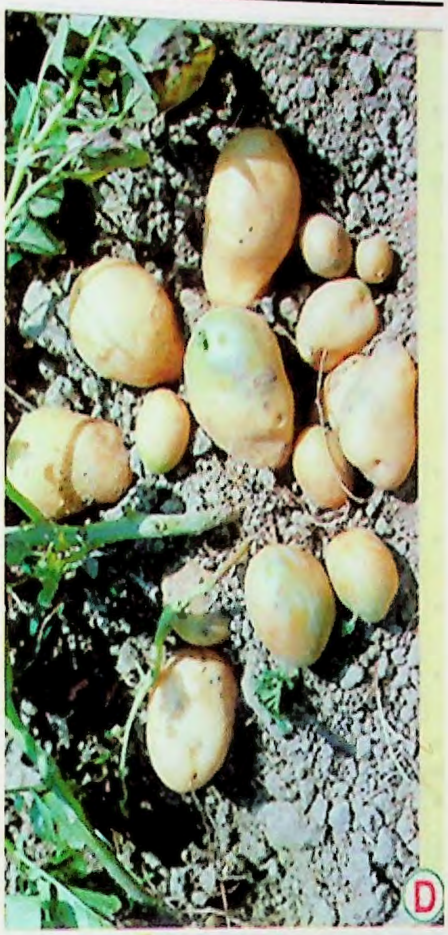


PLATE 8

Chapter - 4

DISCUSSION

Chapter - 4

DISCUSSION

Potato (*Solanum tuberosum* L.) is one of the cheapest and one of the important vegetatively propagated crops of Bangladesh. Potato conquered about 85% among vegetables in the world. "The peasant's staff of life, the gourmet's delight, nutritious, delicious lauded and maligned the paradoxical potato, the amazing spud". Potato has great adaptability to a very wide range of climatic conditions, high yield potential and versatility as a food; hence its production is ever increasing.

In the present days world mankind is faced with a number of gigantic problems in the area of agriculture, which demand urgent attention and immediate solution. This problem is related to food and other requirements. The techniques of plant cell and tissue culture have become popular and useful methods in many temperate countries, which are being applied to the solution of problem in agriculture and forestry (Rao and Lee, 1986). Plant regeneration from tissue culture of potato has been successfully applied to breeding programs by many workers. In this communication we describe a method for rapid multiplication of potato from cultured plant segments. Successful application of tissue culture method involves the establishment of a more or less differentiated cell or tissue under defined culture conditions, proliferation of a number of cells and the subsequent regeneration of plants (Larkin and Scowroft, 1981).

For many years tissue culture techniques have been applied to improve potato production by means of micropropagation, pathogen elimination, *in vitro* tuberization, and germplasm conservation (Roca *et al.*, 1978).

Potato is usually vegetatively propagated and very much susceptible to the number of viral diseases, which cause remarkable decrease in yield. However, meristem culture is one of the important methods to produce virus free stock plants

(Wang and Hu 1982; Martin *et al.* 1955). Whereas, micropropagation does not permit the microclones to be free from viruses. The shoots of a apical meristem and first set of primordial leaves are generally not connected to the vascular system of the plant and therefore, are not contaminated by virus that travel through the vascular system (Prakash and Pierik, 1993; Rahman, 1998). If this explant is carefully excised so as not to contaminated it with sap from more mature leaves or stem tissue and it is placed in culture tube a virus-free plant can be established. Many important horticulture crops (eg. citrus, strawberries, potato, orchids, lady's finger) are routinely free of viral contamination by using this procedure (Prakash and Pierik, 1993; Ali, 1998).

Smith and Murashige (1970) accomplished the first true meristem culture of isolated angiosperm meristem into a complete plant. Before that time it was believed that the isolated shoot apical meristem of an angiosperm could not direct its own development but rather, relied on subjacent primordial leaves and stems tissue (Ball, 1946; 1960; Dodds, 1998). Generally, to establish a virus free one can culture the apical clone plus two or four subjacent primordial leaves. This technique is also valuable for the maintenance of carefully defined stock of specific varieties and varieties in disease-free state. The size of the meristem explant is critical for virus eradication. Often so called meristem tip cultures have failed to eliminate virus infection because the explant contains shoot apices with vascular tissue instead of true meristem (Rahman, 1998; Doods *et al.*, 1992).

This technique, combined with heat treatment (thermotherapy) or chemical treatment (chemotherapy) has proved to be very effective in virus eradication (Towill, 1981; De, 1992). Placing an actively growing plant in a thermotherapy chamber does heat treatment. Using this technique, 80% to 90% virus-free plants have been obtained. Without heat treatment, shoot tips or meristem can be grown on chemotherapeutants added medium for virus eradication (Ali, 1998). Commercial production of virus-free seed potatoes through meristem culture has been a regular practice in many developed countries.

The present investigation was conducted to develop a standard protocol for improvement of potato (*Solanum tuberosum* L.) using different explants. Four genotypes (Diamont, Cardinal, Multa, Lalpakri) of potato were used in the present investigation. The results, which are discussed in following paragraphs with an endeavor to justify them.

4.1 PRIMARY ESTABLISHMENT OF MERISTEM CULTURE

For primary establishment of meristem culture meristems were isolated from shoot tips of 25-30 days old field grown potato plants cv. Diamont and cultured on MS (Murashige and Skoog, 1962) liquid media with different types of growth regulators like BAP, KIN, NAA, IAA, GA_3 either singly or in combination in order to find out the most suitable culture media formulation for primary establish of the cultured meristems. The cultured meristems were considered to be established primarily when they increased in size and became light green in colour. For primary establishment of meristems liquid media with filter paper bridge were used. Use of the liquid culture methods for different crops have been reported (Yee *et al.*, 2001; White,1968; Steward, *et al.* 1969; Walkey, 1986; Miller and Smith, 1969; Rahman, 1998; Ali, 1998).

Various concentrations and combinations remarkably influenced in resuming growth of meristem. Among the various concentrations and combinations of growth requlators KIN + GA_3 combination showed the best performance in this respect. KIN +GA_3 at a concentration ranging from 0.1 mg/l - 1.0 mg/l were used for assessing the optimum concentration. Among the different treatments MS medium fortified with 0.1 mg/l KIN and 0.5 mg/l GA_3 was found to be most effective in increasing the size of meristem and highest 82% of cultured meristems showed response after 4-5 days of culture without formation of any callus at the base of the explants in most of the media formulations like explants showed shoot formation but in some media formations like BAP, BAP+NAA, KIN+GA_3 the explant produced booth shoot and roots.

4.2 SHOOT MULTIPLICATION

4.2.1 Medium selection

For shoot multiplication established meristems were sub-cultured onto MS semi-soild media supplemented with different concentrations of Auxin, cytokinin and gibberellic acid either singly or in combination in order to find out suitable culture media. Shoot induction and shoot elongation were found to be highly influenced by the type and concentration of growth regulators present in proliferation media.

Among all the combinations and concentrations BAP+GA_3 showed the best performance for shoot multiplication. The maximum 85% of cultured explants induced shoot proliferation in media having BAP 0.5 mg/l + GA_3 0.5 mg/l. The results also demonstrated that the highest mean number (4.3) of shoots per explant and highest length (9.6 cm) of the longest shoot were recorded in media having BAP 0.5 mg/l+ GA_3 0.5 mg/l). Many earlier workers also got satisfactory results in potato crops in BAP - GA_3 combination (Kassains, 1957; Siddique, 1991; Miller *et al.*, 1985; Hussey and Stacey, 1989).

4.2.2 Effect of carbon source

Ongoing investigation showed that different types of carbon sources *viz.* local sugar, sucrose, glucose and fructose and their concentrations exerted differential effect on growth and proliferation of shoots. Among the four carbon sources sucrose at 3% level was found to be the best as carbon source and fructose was also found to be suitable. Normal shoot proliferation and growth of cultured shoots occoured in medium fortified with 20 or 30 or 40 g/l sucrose and 30 g/l sucrose concentration was found to be most effective for shoot proliferation. Superior effect of sucrose as a carbon source other than glucose, fructose, lactose etc. on proper *in vitro* growth and proliferation of different plant species was also reported by many researchers (Bhojwani and Razdan, 1983; Amin and Jaiswal, 1989) in different plant species.

Jarret *et al* (1980) also observed the inhibitory effect of high sucrose concentrations on *in vitro* potato shoot regeneration from potato tuber disc callus. They found 3% sucrose was the optimum concentration for shoot formation.

4.2.4 Effect of number of explants per bottle

An increase in the number of explants per bottle (12×5 cm) increased the number of shoots harvested due to the increase in initial number of axillary meristem.

Lalpakri, under optimum conditions, produced 17 shoots per bottle after 4 week of subculture when 6 explant were cultured per bottle. Optimum conditions referred to the treatment that produced the maximum number of shoots after 4 weeks of culture. This optimum treatment consisted of 3% sucrose and 6 explants per bottle. The shoots produced under optimum conditions were used for the calculation of multiplication rate.

The multiplication rate was calculated on a comparable basis to compare with the multiplication rate of Wang (1977); Goodwin *et al.* (1980a); Hussey and Stacey (1981).

4.3 ROOT INDUCTION

4.3.1 Selection of growth regulators

Root initiation and healthy growth are essential for the *in vitro* shoots. The shoots (>3 cm length) regenerated from meristematic explants of potato were needed to induce roots for their ultimate establishment to the field.

But it may be mentioned that the explants produced both shoots and roots in the same medium in some cases. Percentage of root formation and mean number of roots/shoot were highly influenced by the concentration of IBA. In the present findings MS+(0.05 mg/l IBA + 0.05 mg/l GA_3), was proved most efficient for rooting. Efficient effect of IBA for root induction was also observed by Goodwin *et al.*

(1980b). Among the different concentrations and combination 0.05 mg/l IBA + 0.05 mg/l GA_3 and IAA 0.05 mg/l + 0.05 mg/l GA_3 were found to be the most suitable for shoot length increment but the frequency of rooting was low and roots were very weak. Thes results were also demonstrated by Goodwin *et al.* (1980 a). The adventitious root formation is an energy requiring process and presence of certain level of sucrose is essential for optimum rooting. For root induction different concentrations (10-70 g/l) of sucrose were used. Among the various concentrations 30 g/l sucrose was found to be most favourable for rooting.

Among the different potato cvs. Diamont, Cardinal, Multa and Lalpakri better toot induction was observed for cvs. Cardinal Diamont and Multa. On the other hand Lalpakri was found to be less responsive for rooting.

4.4 *IN VITRO* TUBERIZATION

Potato tuberization is not restricted to underground stem (stolon); every axil of the plant can produce tubers. This character has been manipulated to produce *in vitro* tubers. Morphologically tubers found *in vitro* are same as other tubers. *In vitro* tubers can be sessile on the nodes of the stem (Catchpole and Hillman, 1969; Mes and Menge, 1954) or can be axillary or terminally formed on new growing shoots (Barker, 1953; Hussey and stacey, 1981; Palmer and Smith, 1969b; Stallknecht, 1972; Wang and Hu, 1982). The size of the *in vitro* produced tubers are usually one cm or less (Chapman, 1958; Stallknecht and Farnsworth, 1982) but larger sized tuber have also been reproted (Lawrence and Bariker, 1963; Wang and Hu, 1982). For this reson we have termed *in vitro* produced tubers as "Microtubers".

For microtuber induction nodal segments of *in vitro* multiplied shoots derived through meristem culture of four cultivars were used. The explants were sub-cultured on MS medium with high concentration of KIN, BAP, sucrose and were incubated in different photoperiods (0, 8, 24 h) at 18 $\pm 1^0$C.

4.4.1 Effect of cytokinin

Kinetin is required only if tuberization occurs in the absence of light. The optimum kinetin concentration for dark tuberization was 8 mg/l. A cytokinin requirement for *in vitro* tuberization under continuos dark has been recognized by other research workers (Mingo-Castel *et al*, 1974, 1976; Palmer and Barker, 1973; Palmer and Smith, 1970; Tizio and Biain, 1973) and concentration has varied from 4 to 11 mg/l.

Increased kinetin concentrations from 6 to 10 mg/l decreased the number of microtuber in Cardinal and Multa. In Diamont and Lalpakri the response to increasing kinetin tended to be quadratic. High tuberizaiton percentage without any exogenous regulator has been reported also by Hussey and Stacey (1981). They found in their *in vitro* shoot cultures under long days and continuous light after 3 to 4 months of subculture, tubers were formed even though no growth regulator was added to the culture media.

Microtubers were also induced *in vitro* grown shoots in test tube by increasing the concentration of BAP. The optimum BAP concentration in dark tuberization was 12 mg/l. Increasing BAP concentrations from 2 to 12 mg/l increased the number of tubers in Diamont. This finding was also similar to Hussey and Stacey (1981).

4.4.2 Effect of photoperiod

The photoperiod studies (Table 13) showed that longer photoperiod increased microtuberization in Diamont in all levels of kinetin. With kinetin, short day showed better response than continuous dark. The 24 hour photoperiods produced significantly more tubers than 0 and 8 hour potoperiods at all levels of kinetin. The effect of photoperiod on *in vitro* tuberization was also noticed by Hussey and Stacey (1981) in their *in vitro* shoot cultures. They observed that microtubers were formed in cultures incubated in 16 and 24 hour photoperiods and not in 8 hour photoperiods after 3 to 4 months of subculture. However, Wang and Hu (1982) reported that more tubers were produced under 8 hour photoperiods than 16 hour photoperiods.

4.4.3 Effect of sucrose on *in vitro* tuberization

The results showed that there was no significant difference between 5, 6 and 7% sucrose but on the average, 6% sucrose produced more microtubers than 2, 3, 4 and 8%. Many research workers also found that the optimum sucrose concentration for *in vitro* tuberization was between 6% and 8% (Catchpote and Hillman, 1969; Lowrence and Barker, 1963; Obata-Sasamoto and Suzuki, 1979 b; Palmer and Smith, 1970; Stallknecht and Farnsworth, 1979; Wang and Hu, 1982).

Increasing the sucrose concentration from 2% to 8% increase earliness and percentage of *in vitro* tuberizaiton. According to Lo *et al* (1972) this kind of response was a response to sucrose as an energy source and not as an osmotic modifier since the sucrose effect could not be replaced by mannitol.

4.5 DORMANCY OF MICROTUBER

The length of the dormant period not only depends on cultivar and kinetin concentration, but also on the method of measuring dormancy. The common methods of measuring dormancy in potato is from harvest to sprouting (Coleman and Coleman, 2000; Boguchi and Nelson, 1980; Brunisma and Swart, 1970; Burton, 1963). However, Cho, *et al* (1983) have measured dormacy from planting to tuber initiation to sprouting.

Cardinal tended to have a shorter (6 weeks) dormancy and Diamont, Lalpakri a longer (8 weeks). The media with 8 mg/l level of kinetin shortened (7 weeks) the dormant period for all cultivars. Boguchi and Nelson (1980), using dormancy experiments with regular tuber, found that Norland had a shorter dormancy (40 days) and Russet Brrbank had longer domancy.

The time of emergence of sprouting microtubers averaged from 10 to 17 days. The rate of emergence of sprouted tubers depends on sprout length (Emilson, 1949; Hardford, 1961; Moorby and Milthorpe, 1975) soil temperature (Hardford, 1961; Mooby, 1978), soil moisture (Letnes, 1958; Morris, 1966) and mineral nutrition (Morris, 1966; Moorby, 1978).

4.6 COMPARATIVE FIELD PERFORMANCE

4.6.1 Morphological and yeild performance

Under present investigation, morphological characters and yeild performance of three propagation sources *viz.* tuber, microshoots and microtubers of four potato cultivars *viz.* Diamont, Cardinal, Multa and Lalpakri were observed at different growing stages. For all these cultivars, plants obtained from seed tuber had a greater number of stem than plants from microculture (microshoot, microtuber). Microcultued produced plants gave single stem while tubers produced plants gave multi stemmed plants. In addition most of the plants from microtubes was bigger than plants from microshoot. This finding is similar to Wattimena *et al.,* 1983. Goodwin and Brown (1980) counted the branches as stems, thus they found that plants from tuber pieces and microcuttings did not differ in stem number.

In all potato cultivars *viz.* Diamont, Cardinal, Multa and Lalpakri micropropagated (microshoot, microtuber) plants produced more tuber number than seed tuber produced plants. Between microshoot and microtuber produced plants, plants obtained from microshoots produced more tuber number than microtuber produces plants. Many workers got similar results in tuber yield for different potato varieties (Holiday; Houghland and Akeley, 1959; Goodwin and Brown, 1980).

Between microculture and tuber produced plants tuber mean weight showed a negative relation with number of tubers and weight of tuber. Highest number of tuber was found from microculture (microshoot) produced plants but highest tuber weight was found in tuber produced plants. In addition between microshoot and microtuber produced plants, plants obtained from microshoots showed greater tuber weight than microtuber produced plants. An increase in number of tubers has been reported to decrease the tuber mean weight (Bishop and Wright, 1959; Holiday, 1960; Nelson, 1967).

4.6.2 Disease reaction

Disease reaction was visualized throughout the growing period. Among the three propagation sources (tuber, microshoot, microtuber) for all cultivars viral and bacterial contamination rate of tuber produced plants was greater than the microculture (microshoot, microtuber) produced plants. Only 2.1-4.7% viral infection and 1.0-2.7% bacterial infection were observed in microculture produced plants at 60 days after plantation. Between microshoot and microtuber produced plants greater frequency of viral and bacterial infection was observed in the plants obtained from microshoots.

Potato caused by *Phytophthora infestans* remains a major disease that can rapidly destroy a crop in the field. To produce the disease free (late blight) potato plants meristem culture technique was used. Among the three propagation sources (tuber, microshoot, microtuber) for all cultivars foliar late blight response of tuber produced plants were greater than the microculture (microshoot, microtuber) produced plants. Only 1.2 to 5.5% late blight response was observed in the plants obtained from microculture produced plants. Between microshoot and microtuber produced plants frequency of late blight response of microshoot produced plants was greater than microtuber produced plants. Many earlier researchers had got similar and dissimilar results in diseases reaction for different varieties of potato (Platt, 1992; Slack, 1988; Wright, 1988).

The aim of this study was to standerdize an efficient and reproducible methods for regeration of disease free potatoes. Meristem culture techniques and *in vitro* tuberization have potential for clonal prpagation and also production of disease free seed potatoes.

Chapter - 5

SUMMARY

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SUMMARY

The present investigation was undertaken with a view to establish a standardization for large scale *in vitro* propagation of disease free potato seeds through meristem culture and *in vitro* tuberization. In this investigation four potato varieties *viz*. Diamont, Cardinal, Multa and Lalpakri were tested.

Apical meristem with 2-3 primordial leaves isolated from 25-30 days old filed grown potato plants cv. Diamont were cultured onto filter paper bridge in liquid MS medium supplemented with different concentrations of cytokinin singly or in combination with auxin or GA_3. Among 29 media formulation MS+ 0.1 mg/l KIN + 0.5 mg/l GA_3 was found to be the best formulation for primary establishment of meristem culture.

Established meristems were subcultured onto MS semisolid basal medium supplimented with cytokinin, auxin and gibberellic acid either singly or in combination. Among 22 media formulation the suitable media composition for shoot proliferation from established meristems were 0.5 mg/l BAP + 0.5 mg/l GA_3, 0.5 mg/l BAP+1.0 mg/l IBA and 0.1mg/l BAP + 0.1mg/l GA_3. Among these three suitable media formulation media with 0.5 mg/l BAP + 0.5 mg/l GA_3 found to be the best for shoot proliferation. Among the four carbon sources (local sugar, sucrose, glucose, fructose) sucrose was found to be the best for shoot proferation for nodal segments of *in vitro* derived shoots. For all four cultivars media containing 3% sucrose was found to better than 6% sucrose for shoot proleferation. Among the four cultivars of potato *viz*. Diamont, Cardinal, Multa and Lalpakri, higher frequency of shoot proliferation was observed in cv. Multa.

For adventitious root induction *in vitro* shoot segments were subcultured on MS medium supplemented with different concentration of auxin singly on in combination. Among the 16 media formulation 0.05 mg/l IBA+0.05 mg/l GA_3 in MS medium was appropriate media composition for adventitious root induction for all four cultivars. The frequency of rooting was also influenced by the sucrose

concentrations. Among seven concentrations (10-70 g/l) of sucrose media with 20-40 g/l of sucrose were enough for adventitious root induction. Low concentration (10 g/l) and high concentration (70 g/l) of sucrose were not suitable for root induction.

Microtubers were induced on meristem derived *in vitro* grown shoots in test tube by increasing the concentration of BAP, KIN and sucrose in the MS culture media. KIN showed better performance than BAP for microtuber induction and among the six concentrations (2-12 mg/l) of KIN, 8 mg/l KIN was the most effective and more preffered concentration for microtuber induction. By increasing the concentration of sucrose increased the frequency of *in vitro* tuberization. The media containing 60 g/l sucrose was found to be the best among seven concentrations (20-80 g/l) of sucrose for microtuber induction for all four potato cultivars.

The photoperiod studies for microtuber induction showed that longer photoperiod condition was better for *in vitro* tuberization at all levels of kinetin for all four cultivars. With kinetin, short day photoperiod gave a response similar to continuous dark. The 24 hour photoperiods produced significantly more microtubers than 0 (continuous dark) and 8 hour photoperids at all levels of kinetin.

The measuring dormant period of microtubers from harvest to sprouting, the kinetin level of 8 mg/l showed shortened the domant period for all four cultivars. A period of 18 days was required for Diamont, Cardinal and Longer period (+23 days) for Multa.

Comparative field evaluation results indicate that among three propagule sources (tuber, microtuber, microshoot) maximum tuber weight was found in tuber produced plants for all four cultivars. But plants from microculture (microshoot, microtuber) showed a greater number of tuber than tuber produced plants. Between the two microculture (microshoot, microtuber) produced plants microshoot derived plants showed better performance than microtuber derived plants.

Evaluation of disease reaction indicate that plants obtained from meristem derived *in vitro* microculture (microshoot, microtuber) were free from virus, bacteria and fungus (*Phytophthora infestans*), and microculture produced plants of four potato varieties were more vigorus than their source (tuber) plants.

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LITERATURE CITED

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LITERATURE CITED

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Appendix

APPENDIX - 1

Preparation of stock solution of MS (Murashige & Skoog, 1962) basal medium

Constituents	Amount (mg/l)	Strength of stock solution	Volume of stock solution (ml)	Amount for stock solution (mg)	Amount (ml) for 1 litter medium
Stock solution I					
1. NH_4NO_3	1650	20 X	1000	33000	20
2. KNO_3	1900	20 X		38000	
3. KH_2PO_4	170	20 X		3400	
Stock solution II					
1. $MgSO_4.7H_2O$	370	20 X	1000	7400	20
Stock solution III					
1. $CaCl_2.2H_2O$	440	20 X	1000	8800	20
Stock solution IV					
1. $FeSO_4.7H_2O$	27.8	20 X	1000	556	20
2. $Na_2EDTA.2H_2O$	37.3	20 X		746	
Stock solution V					
1. $MnSO_4.4H_2O$	22.3	20 X	1000	446	20
2. H_3BO_3	6.2	20 X		124	
3. $ZnSO_4.7H_2O$	8.6	20 X		172	
Stock solution VI					
1. KI	0.83	1000 X	200	830	2
2. $CuSO_4.4H_2O$	0.025	1000 X		25	
3. $NaMoO_4.2H_2O$	0.25	1000 X		250	
4. $CoCl_2.6H_2O$	0.025	1000 X		25	
Stock solution VII					
1. Myoinsositol	100	100 X	100	1000	2
2. Nicotinicacid	0.5	100 X		5	
3. Pyridoxine HCl	0.5	100 X		5	
4. Thiamiine HCl	0.5	100 X		5	
5. Glycine	2.0	100 X		20	

Dissolve $FeSO_4$, $7H_2O$ and Na_2.EDTA. $2H_2O$ separately in 175 ml distilled water by heating and constant stirring. Two solutions were mixed, the pH was adjusted to 5.5 and then distilled water was added to make up the final volume to 1000 ml.