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A Biotechnological Strategy to Improve Indigenous Potato Cultivars of Bangladesh with Enhanced Starch Content

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A BIOTECHNOLOGICAL STRATEGY TO IMPROVE INDIGENOUS POTATO CULTIVARS OF BANGLADESH WITH ENHANCED STARCH CONTENT

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BY

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JUNE 2011

DEDICATED

TO MY

PARENTS

DICLARATION

I hereby declare that the whole work submitted as a thesis entitled "**A Biotechnological Strategy to Improve Indigenous Potato Cultivars of Bangladesh with Enhanced Starch Content**" in the Department of Botany, Rajshahi University, Rajshahi, for the degree of Doctor of Philosophy is the result of my own investigation. The work was carried out under the supervision of Professor Dr. M. Monzur Hossain, Department of Botany, Rajshahi University, Rajshahi. The thesis has not already ready been submitted in the substance for any degree and has not been concurrently submitted in the candidature for any other degree.

Mohammad Mahbube Alam
28/06/2011

June 2011 (Mohammad Mahmube Alam)

CERTIFICATE

It is my pleasure to certify the thesis entitled "**A Biotechnological Strategy to Improve Indigenous Potato Cultivars of Bangladesh with Enhanced Starch Content**" submitted by Mohammad Mahmube Alam, Plant Breeding and Gene Engineering Laboratory, Department of Botany, Rajshahi University, Rajshahi, Bangladesh for the degree of **Doctor of Philosophy**.

I hereby certify that (i) the candidate has fulfilled the residential requirements, (ii) works embodied in the thesis were carried by the candidate and (iii) the data, to the best of my knowledge are genuine and original. No part of the work has been submitted in the substance for any other degree.

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ABSTRACT

Present study was under taken with a view to improve the yield potential of indigenous (local) potato varieties (IPVs). Six IPVs viz., Lal Shill, Patnai, Shill Bilati, Lal Pakri, Sada Gutti and Challisha were selected as experimental materials. These IPVs are popular among the potato growers of the Northern part of Bangladesh because of their high value due to high culinary quality and storability. However, tuber yield of these IPVs substantially decreased. They are cevierly infected with different viral diseases.

In the present study three *in vitro* techniques viz., i) heat treatment followed by meristem culture for the development of virus free planting materials; ii) induction of microtuber for rapid production of virus free planting materials and iii) induction and evaluation of somaclonal variation were employed for the varietal improvement of the selected IPVs.

For the development of virus free planting materials, shoot tip explants of five weeks old tuber grown shoots of six IPVs were cultured on to MS_0-agar medium and incubated in an incubator in dark at three different temperature regimes (37°C, 42°C and 47°C) and incubated for 10, 5 and 3 days, respectively. The shoot tip cultures were then transferred in a plant growth chamber and incubated for three weeks at 22°C in 16h/8h light/dark condition. The meristems were isolated from the shoot tips of *in vitro* grown plantlets. Individual meristem derived plantlets were carefully multiplied by node cutting and analyzed for potato virus X(PVX), Y(PVY) and potato leaf roll virus (PLRV) through ELISA.

Thermal shock of *in vitro* grown plantlets at 47°C followed by meristem culture was found to be the best technique for virus elimination among tested IPVs. It was found that virus eradication from the indigenous varieties was very difficult. Shoot multiplication into MS semisolid basal medium supplemented with different cytokinins, auxins and GA_3 either singly or incombination resulted that media formulation with 0.5 mg/l BAP + 0.5 mg/l GA_3 found to be the best media formulation for meristem culture establishment and subsquent shoot proliferation. Among the carbon sources sucrose was found to be the best and 3% sucrose was better than 6% sucrose for meristem derived shoot proliferation. The tuber yield/plant of *in vitro* produced plantlets in the field was much better than the tuber grown

plants. Furthermore, *in vitro* produced plantlets of all the indigenous varieties having normal plant height produced more but smaller tubers/plant than the tuber derived plants.

The aim of next part of the present investigation was to establish a standardized protocol for large scale *in vitro* propagation of virus free planting materials through *in vitro* tuberization for four IPVs (Lal Shill, Patnai, Shill Bilati and Lal Pakri). Microtubers were induced *in vitro* to meristem derived shoots by manipulating the concentration of BA, KIN and sucrose in MS medium. Between two cytokinins, KIN showed better performance than BA in microtuber induction. Among the six concentrations (2-12 mg/l) of KIN, 10 mg/l KIN was the most effective and more preferred concentration for microtuber induction. In continuous dark it was observed that increase the level of KIN also increased the percentage of tuberization and number and weight of microtuber/shoot. By increasing the concentration of sucrose increased the frequency of *in vitro* tuberization. The media containing 60 g/l was found to be the best among seven concentrations (20-80 g/l) of sucrose tested followed by 70 g/l sucrose for microtuber induction for all four IPVs.

Assessment of the effect of photoperiods on microtuber induction showed that longer photoperiod condition was better for *in vitro* tuberization at all levels of KIN for all four IPVs. Short day photoperiod gave a response similar to continuous dark. The 24 h photoperiods produced significantly more microtubers than 0 (continuous dark) and 8 h photoperiods at all levels of KIN. The microtubers induced to develop in 8 mg/l KIN supplied medium had shorter dorment period (time taken from harvesting to sprouting). Field evaluation results indicated that among four IPVs higher tuber weight was found in IPV Patnai.

The third part of this study was carried with a view to optimize culture media formulation for callus induction and subsequent plant regeneration in four IPVs (Lal Shill, Patnai, Shill Bilati and Lal Pakri). The callus derived plantlets of four IPVs were transplanted on to field and possible occurrence of somaclonal variation was evaluated. Two types of explants taken from *in vitro* shoots of four IPVs were cultured on to MS medium supplemented with various auxins and cytokinins. It was observed that 2,4-D was the best auxin when used singly for callus induction in potato and 2.5 mg/l was found to be the most

effective in callus induction. Between two types of explants, internodal explant was found more responsive than leaf explant for callus formation.

The combinations of BA+NAA and KIN+NAA combination was found more effective for shoot regeneration for internodal and leaf explants derived calli. BA+NAA combination was found to be more effective than KIN+NAA for maximum shoot induction from leaf explants derived calli. Among various treatments, the media fortified with 3.0 mg/l BA+0.1 mg/l NAA and 3.0 mg/l BA+1.0 mg/l NAA were found to be the best formulations. The highest 50% calli derived from internode explants induced to develop multiple shoots regeneration in medium having 4.0 mg/l KIN+1.0 mg/l NAA. Plants regenerated through callus culture displayed pronounced somaclonal variations for plant height, number of leaves/plant, number of tubers/plant, tuber weight/plant and starch content in tuber. Some of the somaclones showed promisingly higher amount of tuber yield per plant with enhanced starch content in the tuber. This variation may be useful for varietal improvement of these IPVs, but further study is needed for this.

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Chapter I

GENERAL INTRODUCTION

1.1. SIGNIFICANCE OF POTATO

Potato (*Solanum tuberosum* L.) is a tuber yielding plant, which serves as a multiuse horticultural vegetable crop. It is the world's fourth most economically important food crop, after wheat, rice and maize. It has high nutritional value and great yield potential. Potato provides roughly half of the world's annual production of all root and tuber-based food, making it the leading non-cereal crop. It is a part of the diet of half a billion consumers in the developing countries (Ghislain *et al.* 1999). Potato is grown not only for food, but also for animal feed, industrial uses and seed tuber production.

The potato crop is 83% more efficient than rice in producing protein. The nutritional content of potato tubers is extremely high in that the protein produced is made up of a high proportion of essential amino acids. Although limiting in sulphur-containing amino acids such as methionine and cysteine, potatoes do contain amino acids rarely found in other plant protein sources, such as lysine. Potatoes are also a good source of minerals, at least 12 essential vitamins, and extremely high content of vitamin C comparable to other food crops (Struik and Wiersema, 1999). Potatoes have served as raw materials for many different industrial/commercial products, such as flour, starch and alcohol (Zaheer, 1998).

It is grown in different climatological zones, including temperate, sub-tropical and tropical regions, and in very different socio-economic environments, ranging from large-scale farming in the USA to small holdings in Peru. Currently the crop is grown in about 130 countries and covers about 18 million ha per annum. The Russian Federation and Poland together account for 26% of the world's cultivated potato area, followed by India and China, which collectively, account for 25% (Maldonado *et al.* 1998). There is a clear and continuous shift in production of potato from the developed countries to the developing countries (Struik and Weirsema, 1999; Caldiz *et al.* 1999; CIP, 2000). Developing countries in Asia currently account for 24% of global potato production up from 7.6% forty years ago. Potato production and use expanded more in Asia than in any other region of the world

during the last four decades (CIP, 2000). The key factors contributing now and in the future to raising yields in developing countries are improved plant nutrition and disease free planting materials (Ghislain *et al.* 1999). Given these developments, scientists/researchers policy makers have increasingly focused attention on potato production for Asia as an area of anticipated growing importance. Bangladesh produces about 2.2 million tons of tuber crop annually of which potato alone contributes 1.6 million tons with and average production 11.4 tons/ha as against 1.84 tons of rice and 2.23 tons of wheat (BBS, 2007).

1.2. BOTANICAL ASPECT OF *SOLANUM*

Potato belongs to the family Solanaceae, a large group containing many species, which typically can produce underground tubers as a means of propagation (Turner and Evans, 1998). The family Solanaceae contains 84 genera and almost 3000 species that occur on every vegetated continent of the world. The Solanaceae is split into three subfamilies; the Solanoideae, Cestroideae and the Nolanoideae. The Solanoideae contains 52 genera including *Atropa*, *Datura*, *Capsicum* and *Solanum*. The Cestroideae contains 30 genera including ornamental and cash crops such as *Nicotiana*, *Petunia*, *Salpiglossis* and *Schizanthus*, whereas the Nolanoideae contains 2 genera, *Alona* and *Nolana* (**Fig. 1.1**) (Deljou, 1997).

The genus *Solanum* contains approximately 150 tuber-bearing species and is divided in to two sub-genera: *Pachystemonum* and *Leptostemonum*. *Pachystemonum* contains five sections including *Petota*, which consists of two sub-sections *Basarthrum* (non-tuber bearing) and *Potatoe*, which includes the entire tuber-bearing species. It is further sub-divided in to 18 series. The series *Tuberosa* comprises 68 wild and 8 cultivated species including *Solanum tuberosum* and many closely related species (Ward, 1991).

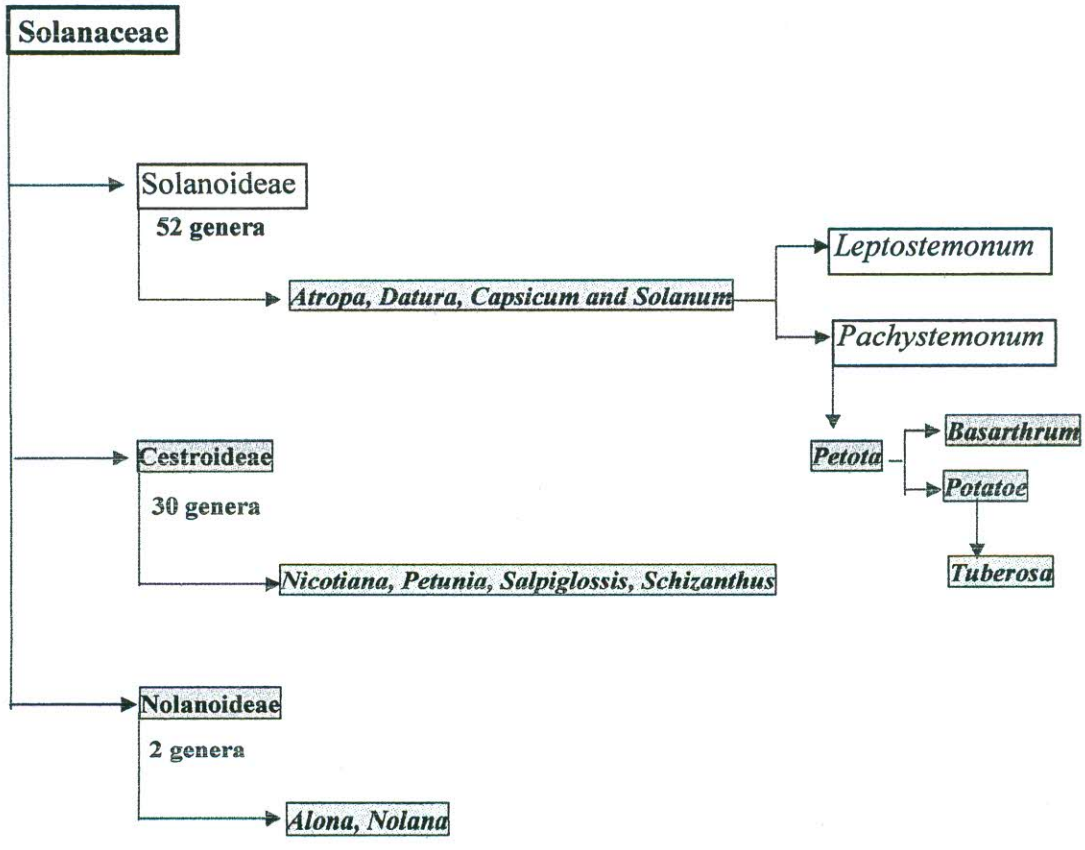
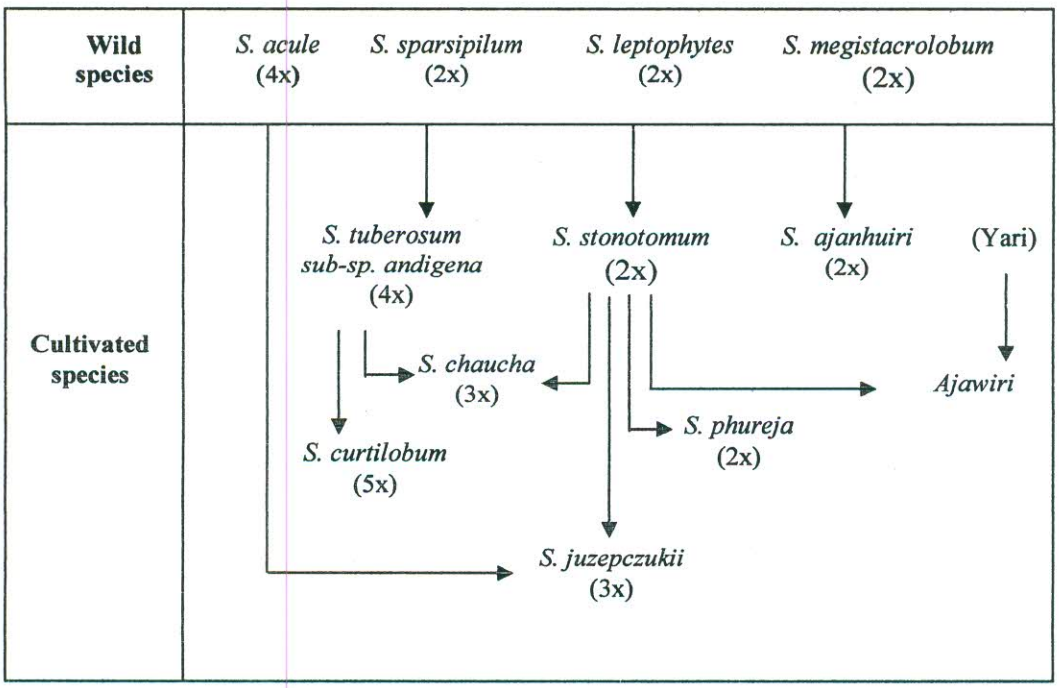


Figure 1.1. Classification of the genus *Solanum*

The CIP maintains the world's largest bank of potato germplasm, including some 1,500 samples of approximately 100 wild-type species and 3,800 traditional Andean cultivated potatoes (CIP Brochure, 2000). The basic chromosome number of Solanacae is $n=x=12$ (Hawkes, 1992). Most (73%) of the species are diploids, very few (4%) are triploids, with 15% tetraploids, 2% pentaploids and 6% as natural hexaploids (Hawkes, 1992). Cytological analyses of interspecies hybrids involving potato have indicated small differences between the constituent genomes. Lester (1965) confirmed very strong serological similarities between *S. acaule* and species in the series *Tuberosa* and parts of *Yungasensia* (including for example, *S. chacoense*).

1.3. THE ORIGIN AND DISTRIBUTION OF CULTIVATED POTATO VARIETIES

There are seven cultivated species of potato including; diploids (*S. ajanhuiri*, *S. phureja* and *S. stenotomum*), triploids (*S. chaucha*, *S. juzepczukii*), tetraploids (*S. tuberosum* sub-sp. *andigena*) and one pentaploid (*S. curtilobum*) (Struik and Wiersema, 1999.). It is believed that four wild species, *S. leptophyes*, *S. megistacrolobum*, *S. sparsipilum* and *S. acaule* were involved in the creation of this group [**Fig. 1.2.** (Hawkes, 1992)].



Source: Bushra, 2002

Figure 1.2. Evolutionary relationships of cultivated potatoes and their ploidy levels

By the mid-eighteenth century, the areas planted with potatoes had begun to increase and many peasant farmers came to depend on the crop because of its ease of cultivation, reasonable yields and good nutritional status. However, the sudden appearance of potato blight (*Phytophthora infestans*) caused severe losses in 1845 and 1846, bringing famine to Ireland and severe hardship to parts of mainland Europe (Turner and Evans, 1998). The famine was followed by a decline in the area under potato cultivation in Europe, with only Britain slightly increasing potato consumption from 88 kg in 1914 to 102 kg per head per year in 2000 (BCP, 2000). Until the middle of the last century, the number of introductions

from South America was limited, so that the genetic base of most breeding programmes that developed commercial cultivars was relatively narrow. The need for more genetic variation in agricultural characters, including resistance to diseases and pests, has stimulated the use of genetic material from either wild or primitive species. As a result, most of the newer S. *tuberosum* cultivars now have gene combinations introgressed from other species (Struik and Wiersema, 1999).

1.4. MORPHOLOGICAL FEATURES OF POTATO

Potato is a herbaceous tuber-bearing plant. The traditional way to propagate potato is by planting seed tubers. Potato plants raised from *in vitro*-grown or from true potato seeds usually have a single stem (Deljou, 1997). Stems are either determinate or indeterminate depending upon the cultivar. The lower part of the stem is always hollow and triangular in cross-section (Struik and Wiersema, 1999). Potato leaf foliage is pinnately compound. Each leaf consists of a terminal leaflet and a few pairs of lateral leaflets. There is usually a pair of secondary leaflets between the two adjacent lateral leaflets. The midrib of the leaf consists of two sections, with the first part (rachis) holding the leaflets and the second part (petiole) connecting the rachis to the stem. At the contact point between the petiole and the stem there are two bracket-like stipules surrounding half of the stem. There are varietals differences in the number, size and colour of leaflets and secondary leaflets. Leaf form can be profoundly changed by daylength and temperature (Cutter, 1992).

Depending upon the potato cultivar, flowers may or may not be produced. Flowering is always accompanied by tuber initiation. Flower colour varies from white to purple and flowers may lead to berries or be aborted, which is either due to varietal differences or to strong self-incompatibility of the flowers (Deljou, 1997). Tubers, which are the underground organs, are botanically swollen stem tissues, since their cross-section shows a typical stem structure, at the end of the stolons. Tubers are highly organised for food storage and vegetative propagation. Tuber formation is a complex physiological phenomenon. It usually takes place in a short period of time (one or two weeks) depending upon the cultivar, similarly, colour and shape of the tubers is genotype dependent. The first step in the formation of tubers is stolon formation (Jackson, 1999; Struik *et al.* 1999). The colour of the cortex of the tubers varies from white, yellow, lemon, red, purple and blue. Stolons normally

develop first at the most basal nodes, and then at progressively higher ones. The number of stolons per stem declines with increasing stems number. The number of nodes which subtend stolons and the length of the stolons are adversely affected by low levels of nutrients such as nitrogen. Irrigation during stolon formation is crucial to the manipulation of stolon number per stem. A much-branched fibrous root system is formed either by the seedling taproot, or by adventitious roots in tuber-bearing plants. In the early stages of growth the root system is restricted to the surface soil, the roots turning downward after extending for some distance horizontally. Potato crop matures within 90-120 days providing small edible tubers within 60 days (Cutter, 1992).

1.5. IMPORTANT DISEASES OF POTATO

Potato is susceptible to a variety of diseases caused by bacteria, fungi and viruses. Further damage can be inflicted by insects such as aphids, cutworms, colorado potato beetles and leaf hoppers. Disease affects a plant in four main ways; either by causing the premature death of foliage; by stunting growth or reduced growth of foliage; by causing a breakdown in the transport of photosynthates to the tubers; or by causing tubers to rot either during tuber growth or during harvesting (Struik and Wiersema, 1999). Some of the economically significant diseases of potato are described here.

1.5.1. **Potato Virus X (PVX)**

Potato virus X is known under a variety of names, including mild mosaic virus, common mosaic virus and potato interveinal mosaic virus (Gleadle, 1992). The virus is found worldwide and infects only potato and tomato. Most strains are very mild and cause only slight yield reduction, whereas, highly virulent PVX strains (capable of causing considerable loss of yield) are no longer prevalent. Transmission of the virus occurs by infectious sap from leaves and tubers (Alam *et al.* 2004). The infection can then be carried on mechanical planters, cultivating equipment, by animals and by contact of sprouts, leaves or roots (Salazar, 1996). Infected cells die very quickly and, since the virus can only exist and multiply within living cells, it dies before spreading through the remainder of the plant. If virus does spread within the plant, however, necrotic spots appear on the leaves around the growing point. Necrosis spreads to the stem and the plant dies back from the top. Death

from top necrosis takes a few weeks. The tubers from infected, necrotic plants have necrosed eyes or may produce plants that show a general necrosis. The necrotic offsprings soon die, though some healthy plants may survive by escape (Gleadle, 1992). Virus can be controlled by the application of insecticides against virus vectors, use of high quality virus free starting material; use of virus resistant varieties; elimination of infection sources within and outside the field and using isolated growing sites (Caldiz *et al.* 1999).

1.5.2. **Potato Virus Y (PVY)**

This potato virus is the type member of potyvirus group and transmitted by vectors directly through aphid contact (Ruize De Galarreta *et al.* 1998). Symptoms of PVY are chronic, and may cause the following changes in the behaviour of potato plant (Salazar, 1996):

i) Macroscopic alterations in stems and leaves; colour deviations (vein clearing, mosaic, yellowing); deviations in shape, size or texture (leaf roll, crinkle, leaf deformation, rugosity, enations); deviation in general appearance of plant (dwarfing, stunting, bunching).
ii) Macroscopic alterations in tubers; deviations in shape, texture (tuber elongation, overgrowth, cracking).

These alterations are often associated with small yield loss. If infection occurs late in the season, plants may even show hardly any symptoms at all. Yet a certain proportion of the daughter tubers of virus infected plants will be infected with the virus and when used as seed tubers, they may cause severe secondary symptoms. Viruses are usually not transmitted from one generation to the next when sexual reproduction occurs. Control measures for PVY include the use of virus free seed potatoes; use of resistant varieties, rouging out of diseased plants early in the season and disruption of landing behaviour of aphids (by using aluminium foil or polypropylene fleece) (Harrewijn *et al.* 1991).

1.5.3. **Late Blight {*Phytophthora infestans* (Mont.) De Bary}**

It is one of the most serious potato fungal diseases that earned a place in history during the Irish famine. It may spread exceptionally quickly both within a field and from field to field and can destroy a susceptible potato crop in only a few weeks (Ruize De Galarreta *et al.*

1998). All parts of the plant can be attacked. The first signs are water soaked lesions on the leaves in cool, wet weather. Lesions appear between 3 and 7 days after infection, are initially light green, and then turn brown. These spread to the petioles and stems and in severe cases, whole plants and field may be killed. Infected tubers are irregular, and develop lumpy areas of various sizes with a purple to brown skin. The contribution of infected seed tubers to its spread is usually minor, since other sources of inoculums are abundant. The fungus is air-borne, seed-borne and soil-borne. It survives on plant debris, refuse heaps or in store (Andersson *et al.* 1998). Due to the rapid multiplication and spread, however, every source of inoculums must be controlled. The spread of blight can be controlled by spraying with suitable fungicides (copper based); by sanitation (destruction of diseased foliage by spraying) preventing spores being washed down to tubers, thus reducing levels of tuber infection (NIVAA, 1996) and by growing resistant varieties.

1.5.4. **Potato Cyst Nematodes (*Globodera pallida* and *G. rostochiensis*)**

Worldwide the most significant nematode problems are the golden and white cyst nematodes (*Globodera pallida* and *G. rostochiensis*) and root-knot nematodes (*Meloidogyne chitwoodi, M. incognita*) (Ruize De Galarreta *et al.* 1998). Nematodes are usually dispersed by wind, irrigation water, with infected tubers or other plant parts (Jeger *et al.* 1996). Nematode larvae (hatch from eggs held in golden or dark brown cysts) penetrate the nearby roots of host plants. Infected plants are stunted, with weak stems and chlorotic or necrotic leaflets. These cause severe yield reduction. The first signs are an intensive branching of the root system, in severe cases, the root system will be small and fibrous with small tubers (Struik and Wiersema, 1999). Control measures include soil surveys (disinfection of soil by use of D-D, EDB, Vorlex or any other nematicides); use of clean seed tubers and clean fields; steam sterilization of contaminated equipment; crop rotation (Asscheman *et al.* 1994) and use of resistant varieties (Gleadle, 1992).

1.6. POTATO IMPROVEMENT

The first improvement of Irish Potato (*Solanum tuberosum* Linn.) probably occurred in prehistoric times. In the forty years period between 1850 and 1890 notable improvement in potato varieties was made in America. The absence of progress has not been to lack of

efforts, although since 1890 emphasis has been placed on clonal selection instead of sexual breeding method, which was practiced during the previous forty years period. The methods employed to secure improved varieties of potato may be conveniently divided in to (a) clonal selection and (b) sexual breeding. These two methods are fundamentally different. Johannsen (1903) considered a pureline to be the progeny of one or more self- fertilization from a single homozygous ancestor. The pure line theory has been extended to cover clonal or asexual propagation in both plants and animals. Johannsen worked with self-fertilized crop and found that selection within such a pure line was of no practical value. While variation occurs within such pure lines, these variations are found not to be inherited. In vegetatively propagated plants the importance of mutation in the production of new types appears to be dependent on the material. The period in which most of the important potato varieties were produced was between 1850 and 1890. A few of the potato breeders during this period practiced hybridization. The most notable of these was Pringle. However, the large majority of the breeders grew seedlings from naturally set seed balls. The heterozygous condition of potato varieties led to sufficient variation in the seedling make this an encouraging line of work. The breeders introduced many promising seedlings into the trade. Sexual breeding has given us our present commercial potato varieties and it is this kind of breeding rather than clonal selection that offer possibilities for further improvement. A study of the possibilities of sexual breeding was made with material obtained during the progress of a potato breeding project which has been carried on at the Minnesota experiment station since 1921.

Research on botanical seed or true potato seed (TPS) at the International Potato Center (CIP) Lima, Peru was started in 1977 and breeding work was attempted to characterize the nature of genetic variability for several important traits, as well as to identify clones with general combining ability (GCA) for producing high yielding and uniform lines adapted to various climate (Sadik and Engels, 1985). Several lines of wide adaptation, developed by this programme, have been outstanding in evaluations conducted in a number of countries.

Cold temperature and long photoperiod is the prerequisite for potato flowering and breeding programme. In Bangladesh research on the use of TPS was initiated in 1980 in-collaboration with the International Potato Center (CIP). After several years of research and promotional

activities it was found that the technology is highly feasible in Bangladesh. During the late part of 1980's attention was given to produce OP (open pollinated) seeds, and later on, hybrid seeds locally by creating artificially long days with the help of incandescent lights. Since 1990 effects have been continued to produced commercial hybrid TPS at the Tuber Crop Research Center (TCRC) of Bangladesh Agricultural Research Institute (BARI) under sodium light.

Development of potato varieties has been affected by the use of the basic methods of introduction, selection and hybridization. Hybridization is considered to be the best method for attainment of positive improvements in the production of potato. An advantage of hybridization in potato is that once a hybrid with desired characters is identified, it can be multiplied vegetatively for long time without any risk of segregation. As potato is a tetraploid ($2n = 2x = 48$) crop, wide segregation is obtained in the F_1 progeny of most crosses or in the selfed progeny of clonal variety (Krantz, 1946). Since potato is a vegetatively propagated crop, commercial varieties are heterozygous and segregation of characters will be found in F_1 generation following hybridization. Clonal selection is therefore practiced in the F_1 generation.

1.7. ROLE OF BIOTECHNOLOGY FOR THE IMPROVEMENT OF POTATO

Biotechnology applications deriving from cell biology, molecular biology and molecular genetics differ among crops; these are influenced by relative amenability to cell manipulation and by the nature of their reproduction systems. Potato breeding is slow when compared to other major food crops. Therefore, it is not surprising that several applications of biotechnology have found a particular fit with the potato crop (Ghislain *et al.* 1999).

Major techniques of potato biotechnology can be divided into two categories:

i) The propagation, storage and dissemination of virus-free plants.

ii) The widening of genetic variability and the introduction of novel traits into potato by somaclonal and protoclonal variation, ploidy manipulations, embryo rescue, genetic transformation and somatic hybridization.

While progress in the area of biotechnology provides new opportunities for potato breeding, most experts agree that biotechnology-based methods will always supplement, but not replace traditional breeding methods.

1.7.1. **Micropropagation of Potato**

Micropropagation of potato remains the widest applied use of plant tissue culture technology. Over the last three decades plant regeneration has progressively become possible from a variety of explants/specialised cells of plant species, including dicotyledonous as well as monocotyledonous species (Anthony, 1999). The application of tissue culture and rapid multiplication techniques in potato seed programmes have now become widespread in both developed and developing countries. The reasons for the rapid expansion for multiplication purposes are obvious:

i) Large numbers of disease-free plants can be produced in a short time.
ii) Aseptic conditions result in hardly any loss due to pests and diseases.
ii) Multiplication can take place in a small space within a strictly controlled environment. Therefore all year-round production is possible.

Rapid *in vitro* multiplication of potato involves following three processes (Lommen, 1995):

a) Meristem tip culture for establishing disease indexed primary material (Ward, 1991).
b) Rapid multiplication of disease indexed primary culture through repeated node cutting (Veramendi *et al.* 1997).
c) Multiplication of disease indexed primary culture through *in vitro* microtuber induction (Hoque *et al.* 1996).

1.8. RATIONALAE AND OBJECTIVE OF THE PRESENT WORK

Potato is used as major vegetable crop in Bangladesh and occupying 2nd position after rice. Cultivation of potato in Bangladesh began in the late past of the 19th century. At present potato is grown in about 140 thousand hectares of land in Bangladesh. The variety grown in this country can be categorized into (a) Modern Potato Varieties (MPV) and (b) Indigenous Potato Varieties (IPV). There has been a remarkable increase in the area under potato production from 35 thousand hectares during the last forties to 140 hectares in the recent

years mainly due to the introduction of MPV. Practically the area under IPV did not decline during this period. Rather there has been some increase in the area under IPV and occupies about 36% of the total potato area that accounts for only 27% of the total production (Siddique and Rashid, 1990). Under the present condition, it dose not appear that the importance of IPV will be decrease in near future and indicates the necessity of the improvement of the IPV.

The main difference between MPV and IPV is the composition of starch content and ploidy level (Hossain, 2001). IPVs are either diploid or triploid, contains higher proportion of amylopectin but MPVs are tetraploid with less anylopectin. High amylopectins in IPVs make them sticky and more tasty. In spite of low yields the IPVs are popular among the growers and consumers mainly because it contains higher percentage of dry matter and exhibit good keeping quality under ordinary room temperature. Beside this the IPV gives reasonable yield under low input condition and because of that it fit well in to the production system of small and marginal farmers. Due to farmers and consumers acceptability particular attention should be given to the improvement of the IPV. The low yield of IPV is thought to be due to degeneration from viral and mycoplasmal disease as well as poor management practices (Ahmed, 1982; and Siddique and Hussian, 1988).

The biggest problem of potato in the tropical region is the high temperature during the summer months which makes it almost impossible to store tubers at home for year-round consumption and use as seed due to early sprouting, high respiration rates and rapid breakdown of the food reserve in the tuber (Moorby and Milthorphy, 1975). For vital production, a seed tuber has to be stored under refrigerated condition at 4°C (Anon. 1974; Cho *et al.* 1983) which is lacking in many tropical countries. The alternative low cost technique of storing potatoes in diffused light store has been found very useful under tropical conditions (Callueng *et al.* 1993; Khuyong, 1987; Muyco *et al.* 1990). For this purpose, variety with higher percentage of dry matter is highly desired.

The improvement of IPV has not given proper attention by the different research organizations. Tuber Crop Research Center (TCRC) of Bangladesh Agricultural Research Institute (BARI) mainly concerned with research for the improvement of MPV. However,

TCRC has optimized different cultural practices for cultivation of IPV and yield of some IPV has increased considerably by using improved cultural practices. For the improvement of any crop variation among the population is the most important requirements. Although, the area under potato cultivation is increasing every year, average production per unit area still very low (about 11 tons/ha) compared to many other countries. The increase in the productivity of potatoes is related to the use of quality seed. At present only four to five percent of the total demand of the seed potato is supplied to the formers by Bangladesh Agricultural Development Corporation (BADC) and the remaining demand is met up from farmer's seed. As it is not possible to improve quality of our seed potatoes through import, attention should be given to producing quality seed tuber locally. Moreover, particular attention should be given to the improvement of indigenous potato varieties.

So far limited research has been done for improvement of the IPV apart from improvement of agronomic practice in Bangladesh. Now a days, in many countries of the world virus free potato plantlets are produced by means of meristem culture and/or combination of heat treatment and meristem culture to improve the yield (Kassanis and Varma, 1967 and Dhingra and Sangar, 1983). Very little information on improving the IPV through making them virus free by means of meristem culture is available in Bangladesh. Therefore, it would be logical to apply meristem culture technique to improve the indigenous varieties.

In Bangladesh no work has been carried out so far for the improvement of potato by means of sexual breeding. Because most of existing potato germplasms available in Bangladesh induces develop flower in long day condition. However, in Bangladesh potato is being cultivated during winter season when day-length is short (12 h or less than 12 h) i.e. unfavorable for flower induction. Therefore, Tuber Crop Research Center at Bangladesh Agriculture Research Institute has been focusing varietal improvement through introduction of exotic materials/germplasms.

Somaclonal variation among regenerated plants from callus and protoplast culture has been suggested as a useful source of potential valuable germplasm for plant breeding. The major benefit of somaclonal variation is to create variation in adapted genotypes. Recognition of new genotypes at the whole plant level and their efficient exploitation would however be

very useful breeding programme. For the improvement of potato crop Shepard *et al.* (1980) suggested that it would be more profitable to improve a popular variety selectively rather than to create new one. New potato varieties with both late and early blight resistance were developed through induction of somaclonal variation and subsequent screening of the somaclones (De, 1992).

Keeping in mind the above mentioned aspects of potato breeding the present study was undertaken for achieving the following objectives:

1. Improvement of planting materials through meristem culture and establishment of a cost-effective rapid micropropagation system for selected IPVs.
2. Induction of somaclonal variation through callus culture and field evaluation of somaclones for the varietal improvement of IPVs.

Chapter II

PRODUCTION AND FIELD EVALUATION OF DISEASE INDEXED PLANTING MATERIALS FOR SELECTED IPVs

2.1. INTRODUCTION

In vegetatively propagated potato once systemically infected with a viral disease, the pathogen passes from one vegetative generation to the next. The entire population of a given variety may, over years, be infected with the same pathogen (Wang, 1977). In Bangladesh, the indigenous varieties of potato, which still play an important role in potato production, have been maintained for several decades, since their introduction. The varieties have now become permanently infected with different viruses. Khan (1981) reported that a single plant of the indigenous potato may be infected with 4 or 5 viruses. The presence of viral diseases is an important reason attributed to low yield of these varieties (Ahmed, 1982; and Siddique and Hussain, 1988). The yield reduction may be up to 75% caused by infection of some viruses (Norris, 1953; Fakir, 1985; and Rashid *et al.* 1986).

There are reports that meristem culture is a good technique for eliminating viruses (Norris, 1953; Morel and Martin, 1955; Pennazio, 1971; Gregorini and Lorenzi, 1974; and Marani and Pisi, 1977). The technique works well for potato virus Y and potato leaf roll virus, but is less effective for potato viruses A, S and X. Several researchers, however, have combined meristem culture with heat treatment to improve results (Stace-Smith and Mellor, 1968; Pennazio, 1971; MacDonald, 1973; Faccioli and Rubies Autonell, 1982; Dhingra and Sangar, 1983; and Brown *et al.* 1988). So far very little work has been done in Bangladesh for improving the indigenous potato varieties by freeing them form viruses through meristem culture and/or heat therapy.

In vitro plant growth depends on the nutrition (Dougall, 1981; Gamborg and Shylurk, 1981; Murashige, 1977) and the environmental (Gamborg and Shylurk, 1981; Murashige, 1977) factors. Hughes (1981) referred to these environmental factors as *in vitro* ecology, where light, temperature and gases are the major elements. Gamborg and Shylurk (1981) divided

the nutritional factors into essential components and optional components. The essential components consist of inorganic salts, a carbon source of energy, vitamins and growth regulators. Optional components include organic nitrogen compounds, organic acids, and complex substances such as plant extracts and juices.

The manipulation of the growth regulator composition and balance is commonly the most successful method of regulating *in vitro* shoot multiplication (Lawrence, 1981). The growth regulator composition and balance is based on Skoog and Miller (1957) concept of auxin and cytokinin ratio organogenesis. A high auxin-cytokinin ratio favors shoot formation. The auxin-cytokinin balance in controlling root and shoot initiation appears to be a general phenomenon among plants (Murashige, 1974 and 1977).

There are two sources of *in vitro* shoots, axillary shoots and adventitious shoots. Axillary shoots are derived from activation and proliferation of existing shoot meristems, while adventitious shoot arise through the induction and proliferation of adventitious meristems directly on parent tissue or via intermediate callus (Hussey, 1980; Hussey and Stacey, 1981; Lawrence, 1981; Minocha, 1980). Proliferation of the axillary bud is the most commonly used method for *in vitro* shoot multiplication. The advantages of this method are faster shoot development and genetic stability (Binns, 1981; Boxus and Druart, 1980; Constantin, 1981; D'Amato, 1975 and 1978; Vasil and Vasil, 1980), even though it has a more limited level of multiplication as compared to the callus method of shoot proliferation (Lawrence, 1981; Murashige, 1974; Vasil and Vasil, 1980).

Enhanced axillary shoot production can now be accomplished with many different plant species. A limited number of plant species appear to require a low level of auxin along with cytokinin to induce the axillary branching (Hussey, 1980); most need only cytokinin (Murashige *et al.* 1974; Murashige, 1977). Theoretically, any plant species that produces axillary meristems and responds well to the available cytokinins can be clonally propagated through micro propagation. Representative examples are strawberry (Boxus and Druart, 1980), apple (Jones *et al.* 1977), gerberra spp (Murashige *et al.* 1974), sugar beet (Hussey and Hepher, 1978), gladiolus (Hussey, 1977), ferns (Lawrence, 1981) and birch (McCown and Amos, 1977).

In vitro shoot production of potatoes can be based on regeneration from callus tissue. Nearly every part of the potato plant can be induced to produce callus and adventitious shoots. The organs commonly used are tubers (Bragdo-Aas, 1977; Jarret *et al.* 1980; Lam, 1975; Marani and Pisi, 1977; Okazawa *et al.* 1967), shoots (Marani and Pisi, 1977), leaf rachis (Roest and Bokelmann, 1976), leaf mesophyl (Roest, 1977) and roots (Vasil and Vasil, 1980). However, potato plants derived from callus tissue may be variable (Roest, 1977), or genetically unstable (D'Amato, 1975). Thus, this method is unlikely to be useful for the propagation of commercially important cultivars or locally adapted cultivars. For micropropagation the axillary bud method of producing *in vitro* shoots is preferred.

To-date very little work has been reported on the field performance of the indigenous potato varieties after cleaning from viruses. Hossain *et al.* (1989) and Hossain (1991) reported, however, that the indigenous potato varieties after cleaning form viruses became viney and bushy with a tendency of producing no tuber or numerous small-sized tubers. This raises the question of urgency of field studies on virus free *in vitro* produced plantlets.

General objective of this part of the present study is to develop a standard protocol for the production of disease indexed seed potato tuber. The specific objectives are as follows:

2.1.1. **Objectives**

i) Production of virus free planting materials (Plantlets) through meristem culture and heat treatments

ii) Rapid micropropagation of disease free plantlets through node cuttings

iii) Evaluation of the disease free plantlets under field condition

2.2. MATERIALS AND METHODS

2.2.1. **Materials**

The experiment was carried out in the Plant Breeding and Gene Engineering Laboratory of the Department of Botany, Rajshahi University (RU), Rajshahi (GPS 24°17'-24°31' N latitude and 88°28'-88°43' E) during the period from 2007–2008. Six indigenous varieties were used in this study. The four indigenous varieties (Shill Bilati, Lal Pakri, Sada Gutti and Challisha) were collected from Burirhat Farm, Agricultural Research Sub-station, Rangpur

and the other two varieties (Lal Shill and Patnai) were collected from the farmer's field of Rajshahi. The presence of different viruses in these varieties was detected by Adgen, UK. ELISA kit and are shown in **Appendix I**. List morphological features of the selected IPVs are given in **Table 2.1**.

2.2.2. Collection and Sterilization of Explant

The selected potato varieties were grown in a net house. One month after emergence, one hundred shoot tips of each variety were collected. The shoot tips were first sterilized in 70% ethanol for thirty second, stirring with magnetic stirrer followed by washing in sterile water three times. The shoot tips were then sterilized by stirring for 15 min. in 0.50% sodium hypochlorite solution containing two to three drops of Tween – 20. The explants were then rinsed three times with sterile water.

2.2.3. Culture of Explants and Heat Treatment

One hundred and fifty single nodes of each variety were excised from sterilized plant samples and placed in test tubes containing 10 ml of MS medium (Murashige and Skoog, 1962) solidifed with 8 g/l of agar. Cultured nodes developed into plantlets in the test tubes and these were subcultured for one-time 7 days prior to heat treatment.

The *in vitro* produced shoots as mentioned above were grouped into three sets for heat treatment. Each set contained thirty plantlets of each variety. Of the three sets of *in vitro* plantlets, one set was incubated at 47°C; another set at 42°C and the rest was incubated at 37°C that served as control. The incubation period was 21 days. After incubation the shoots were taken out and the meristems were excised.

Table 2.1 Characteristic feature of IPVs used in the present study

Sl. No.	Potato vrieties	Chromosome nos. (2n)	Morphological characters
01	Lal Shill	3n (36)	Plant tall, erect; stem few, hairy, pinkish green; leaf structure open and green in colour; tuber oval, round, medium with smooth red skin; eyes deep and prominent, moderate in number, concentrated towards the distal end of the tuber; flower pink.
02	Patnai	2n (24)	Plant tall and erect, stem many and very hairy; leaf structure open and green in colour; tuber oval with smooth radish skin; eyes deep, prominent and distally distributed; flower pink.
03	Shill Bilati	3n (36)	Plant tall and spreading; stem few, moderately hairy and light pinkish green in colour; leaf structure open and green in colour; elongated tuber with smooth shiny, slightly radish skin, deep eyes uniformly distributed on the tuber; flower pink.
04	Lal Pakri	3n (36)	Plant short and spreading; stem few, hairy, pinkish in colour, leaf structure open and dark green; tuber medium, round pinkish with white patches, eyes few not uniformly distributed; flower light pink.
05	Sada Gutti	2n (24)	Plant tall and erect, stem moderately hairy; leaf structure open and green in colour; tuber round, irregular, creamy white, eyes deep, moderate in number more at the distal end; flower pinkish white.
06	Challisha	2n (24)	Plant short and spreading; stem few, very much hairy with light pinkish green in colour; leaf structure semi open and dark green; tuber round, medium, creamy white, smooth skin; eyes very few in number, shallow, distributed unevenly; flower light pink.

2.2.4. **Isolation and Culture**

Dome shaped meristem with two-leaf primodia was dissected out by a heat sterilized blade under a dissecting microscope (20-40x) inside the clean bench. Thirty meristems from each set of *in vitro* heat treated plantlets of each variety were excised. Immediately after dissection the meristems were placed on MS medium (10 ml in each test tube) supplemented with 0.01 mg/l of NAA and 1 mg/l of Kinetin (Wang, 1977). The medium was solidified with 8 g/1 agar and the pH was adjusted to 5.8 before autoclaving, which was carried out at 121°C for 15 min. Cultures were incubated at 22±2°C temperature under 16 h photoperiod. The meristems grew into plantlets. The *in vitro* plantlets regenerated from dissected meristems were subcultured after 60 days of incubation for multiplication by single node cutting. At that time the regenerated plantlets became 3-4 cm in length. For subculturing of regenerated plantlets, MS medium at full strength was supplemented with nicotinic acid (0.5 mg/1), pantothenic acid (2.5 mg/1), pyridoxin (1.0 mg/1), and thiamine. HCL (0.5 mg/1), myo-inositol (100.0 mg/1), sucrose (30.0 g/1) (Janet *et al.* 1993) and solidified with agar (8 g/1). The condition of culture was similar to meristem culture.

2.2.5. **Virus Indexing**

Leaves from the heat treated meristem derived cultures were assayed by ELISA for the same types of viruses that were present in the different potato varieties before heat treatment and meristem culture.

2.2.6. **Shoot Multiplication**

After 4 weeks of incubation when the plantlets attained a height of 7-6 cm micropropagation was started. The plantlets were removed carefully from the test tuber over a petridish using a pair of forceps. In the petridish the leaves from the stem were carefully removed and cut into single node segments. A single node segment having undamaged axillary bud and generally it was 5-6 mm long was cultured. The isolated nodes were transferred to the culture tubers for multiplication. The node cutting was placed on the medium in such a way that is should be not pushed bellow surface of the medium. Single node cutting after incubation at 22 ± 2°C with 16 h photoperiods per day grew rapidly and developed into new plantlets. These plantlets were subcultured as required after every 4 weeks.

2.2.7. **Field Establishment of Virus Free *in vitro* Produced Plantlets**

2.2.7.1. *Materials and methods*

The experiment was conducted at the experimental farm of Institute of Biological Sciences, Rajshahi University during the period from November 2008 to March 2009. The three weeks old *in vitro* produced virus free plantlets of six potato varieties obtained from the Experiment 2.2.6 were planted in the field covered with insect proof net on November 28, 2008. The experiment was laid out in the randomized complete block design with three replications. The unit plots were 100×60 cm in size, which accommodated 30 plantlets at a spacing of 20×10 cm. The crop was harvested at maturity during the month of March 2009.

2.2.7.2. *Raising crops*

The plantlets were watered immediately after transplanting and the beds were provided with shades from 10 a.m. to 3 p.m. until the establishment of the plantlets in the soil. The soil of the experimental plots was specially amended with cowdung and coarse sand (1:1 v/v). Fertilizers (Urea-TSP-MP) were applied at the rate of 75-60-75 kg/ha (Hossain, 1987). The entire dose of TSP, MP and 50% of Urea were applied at the time of land preparation. The rest 50% of urea was applied as top dressing into two equal installments at 35 and 50 days after planting. Intercultural operations such as earthing up, weeding and mulching were done as and when needed. Fungicides and insecticides were applied to protect the crops. The experimental plots were irrigated frequently to maintain optimum soil moisture and also to keep the soil cool.

2.2.8. **Data Collection**

2.2.8.1. *Data collection for meristem culture*

The data on meristem culture were recorded on:

i) Percentage of regenerated plantlet

ii) Percentage of virus free plantlet

3.2.8.2. *Data collection on in vitro shoot multiplication*

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

i) Days required for shooting initiation

ii) Percentage of response

Percentage of explant responded was calculated using following formula;

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

iii) Number of shoots/explant

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

iv) 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.

2.2.8.3. *Data collection for field grown plant*

Ten plants were selected at random from each plot for collection of data on the following characters:

i. Successful plantlet (%)
ii. Days to tuber initiation
iii. Days to maturity
iv. Plant height at maturity (cm)
v. Branch/plant (no.)
vi. Leaf/plant (no.)
vii. Leaf area/plant (cm^2)

viii. Tuber/plant (no.)
ix. Tuber weight/plant (g)
x. Dry matter of tuber (%)
xi. Tuber grade (%)

The plantlets that survived in the field were expressed in percentage according to variety. The time required from planting to tuber initiation was recorded by gentle digging the soil around selected plants. Day to maturity was recorded when the leaves just began to senescing. At the time of harvest, plant heights, number of branches/per plant, number of tubers/per plant, tuber weight/plant were recorded. Before harvest leaves/plant was count and the leaf area/per plant was measured by leaf area mater. Weight of tubers was taken with a weighing balance.

The harvested tubers were graded in the following bulky size grades: i) below 10 mm, ii) 10-20 mm, iii) 20-30 mm, and were expressed in percentage. In determining the dry matter percentage of tuber, the fresh weight of tubers was taken immediately after harvest and than dried in an oven at a constant temperature of 80°C for 72 hours. The dried materials were weighed and the percentage of dry matter of tuber was calculated.

2.2.9. **Data Analysis**

The data recorded for different observations were analyzed statistically.

2.3. RESULTS

2.3.1. **Effect of Heat Treatment on Primary Establishment of Meristem Culture**

The results of the percentage of regenerated plantlets after heat treatment and meristem culture are shown in **Figure 2.1(Appendix II)**. Dome shaped dissected meristem and its successive stages of development are shown in **Plate 1**. At 37°C incubation temperature, the highest percentage of regenerated plantlets was observed in Sada Gutti followed by Challisha and Patnai, while the lowest percentage was observed in Lal Pakri. When incubated at 42°C, the maximum percentage of regenerated plantlets was found in Patnai and the minimum percentage was recorded in Sada Gutti. Furthermore, at 47°C incubation temperature, the highest percentage of regenerated plantlets was observed in the varieties,

Challisha, and Lalshil followed by Patnai, while the lowest percentage was recorded in Lal Pakri.

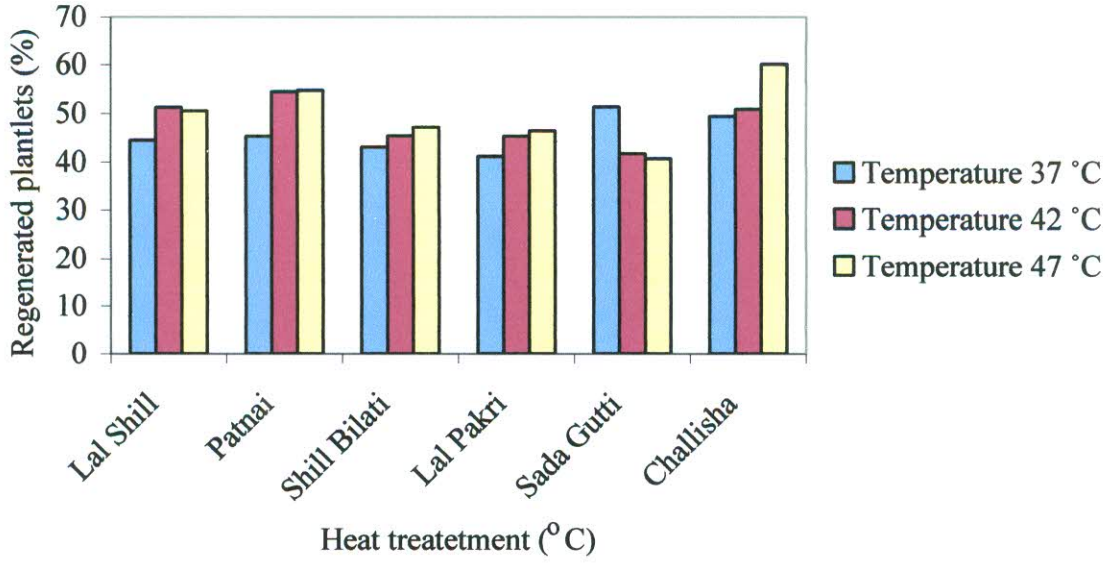


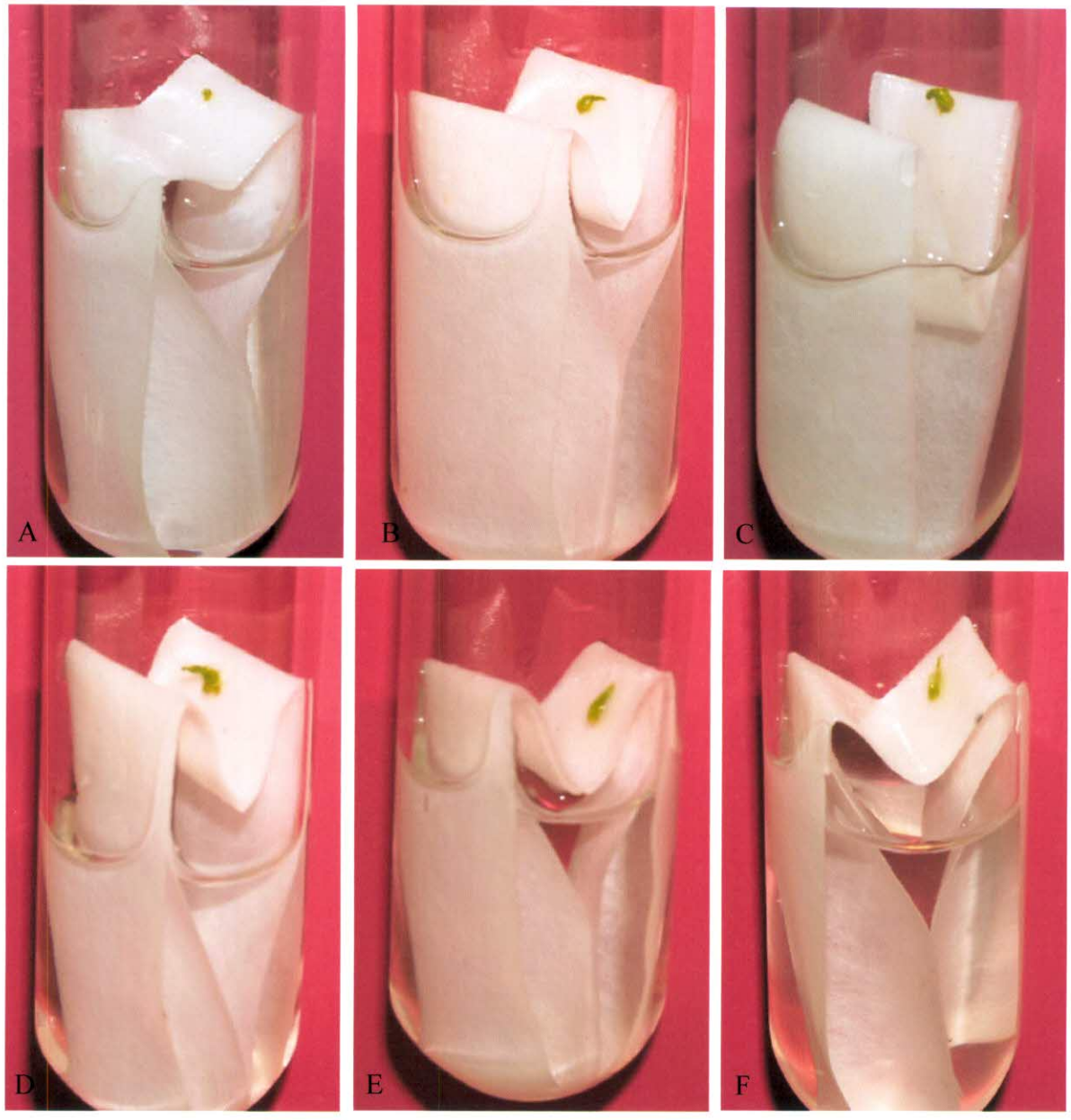
Figure 2.1: Effect of heat shock temperatures on resuming new growth of cultured meristems isolated from six IPVs.

2.3.2. **Genotypic Response of IPVs in Meristem Culture**

Isolated meristems of six potato varieties viz. Lal Shill, Patnai, Shill Bilati, Lal Pakri, Sada Gutti and Challisha were placed separately on "M" shaped filter paper bridge in culture tubes (125 × 25 mm) containing liquid MS medium supplemented with different concentrations and combinations of KIN and GA_3 to find out varietal performance of four potato varieties. Data on days to response, percentage of meristems responded and morphogenic response was recorded after 21 days of culture and is presented in **Table 2.2**.

Days to response ranged from 4 - 9 in all varieties with all four combinations. Among four combinations KIN 0.1 mg/l + GA_3 0.5 mg/l showed better effect on the meristem culture. Among four varieties Patnai showed the best response in media with KIN 0.1 mg/l + GA_3 0.5 mg/l. This variety responded to growth within 4-5 days. Percentage of meristems responded to new growth ranged from 51-78% in all six varieties of potatoes with all combinations of KIN and GA_3 used. Among four combinations, KIN 0.1 mg/l + GA_3 0.5 mg/l was the most

Plate 1



Figures: A-F: Meristem cultures on MS liquid medium supplemented with 0.5mg/l GA_3 of six IPVs:

A. Meristem of IPV Lal Shill, 14 days after inoculation.
B. A developing meristem of IPV Patnai
C. A shoot developed from IPV Shill Bilati
D. A shoot developed from Lal Pakri
E. A deloping meristem of IPV Sada Gutti
F. Initial meristem development of IPV Challisha.

appropriate for growth initiation in four potato varieties and percentage of meristems responding ranged from 56-78%. The highest percentage of meristems responded (78%) was recorded for Lal Shill followed by Patnai (73%) and Lal Pakri (61%). The lowest respond of meristem was recorded for Shill Bilati (56%) with KIN 0.1 mg/l + GA_3 0.5 mg/l.

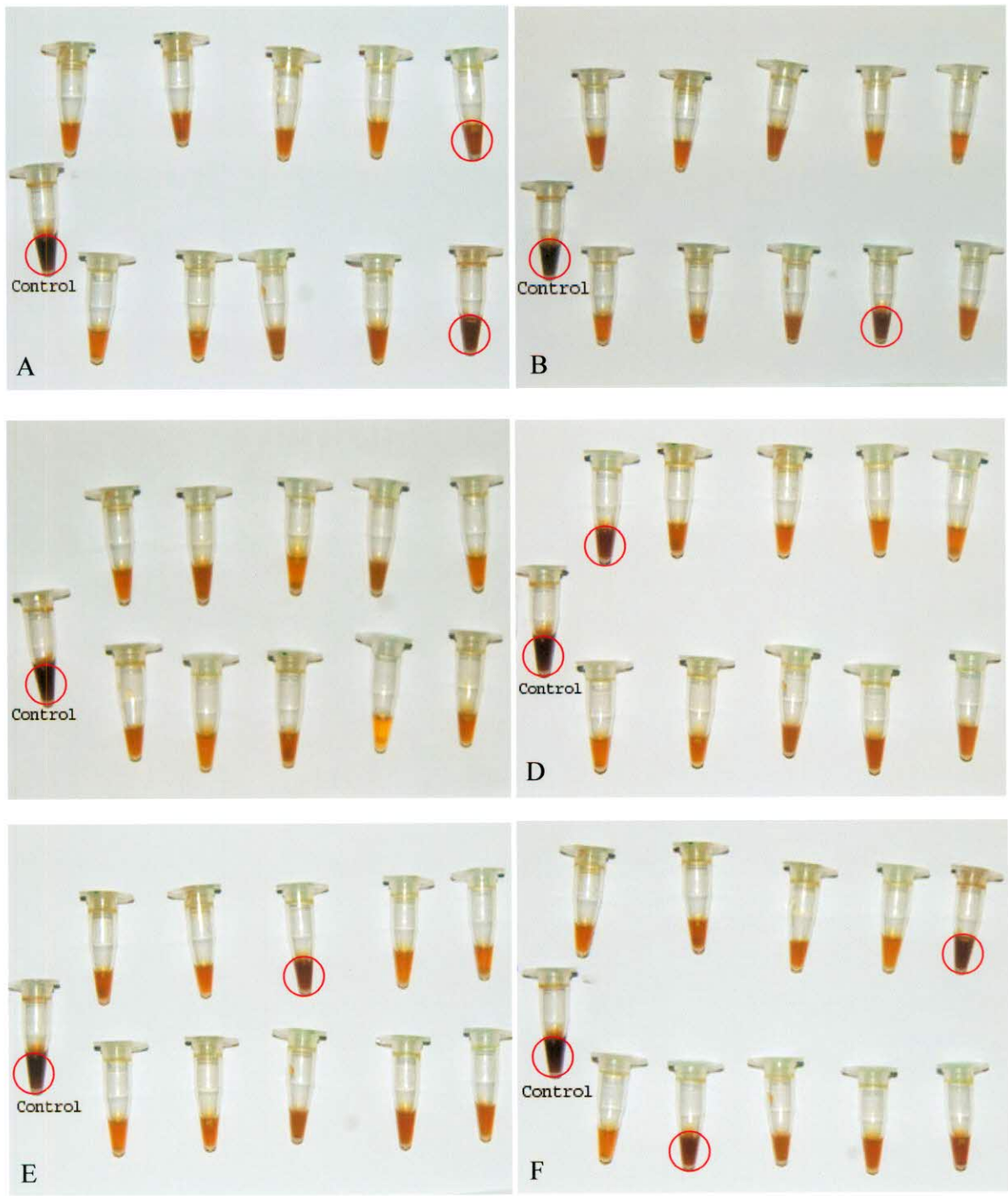
In all combinations of KIN and GA_3 the meristems of four potato varieties produced shoots and roots except in media with KIN 0.5 mg/l + GA_3 0.5 mg/l where Patnai did not produce any root.

In this experiment it was observed that genotypic difference among four potato varieties used in the present experiment responded more or less equally with all combinations of KIN and GA_3 but with KIN 0.1 mg/l + GA_3 0.5 mg/l they showed best performance. Among four varieties, Lal Shill showed the best culture response in all four combinations of KIN and GA_3.

2.3.3. Effect of Heat Treatment on Primary Establishment Virus Free Meristem Culture

Virus free plantlets regenerated from cultured meristem treated at different temperature were tested by ELISA and the results are presented in **Figure 2.2 (Appendix III)** and shown in **Plate 2**. The highest percentage of virus free plantlets at different levels of heat treatment was recorded in Lal Shill followed by Patnai, while the lowest percentage was observed in Challisha. No virus free plant was produced in indigenous variety Shill Bilati and Challisha when 37°C heat treatment was applied to the *in vitro* plantlets. The variety Sada Gutti and Challisha failed to produce virus free plant at 42°C heat treatment. The percentage of virus free plantlets increased with the increase of incubation temperature and the maximum percentage of virus free plantlets was recorded at 47°C incubation temperature in all the varieties.

Plate 2



Figures: A-F: Detection of PLRV virus through ELISA test in heat treated meristem culture derived shoots of six IPVs: Lal Shill (A), Patnai (B), Shill Bilati (C), Lal Pakri (D), Sada Ggutti(E) and Challisha (F). ○ PLRV.

Table 2.2. Genotypic response of six IPVs with different concentration and combinations of KIN and GA_3 in MS liquid medium on primary meristem culture establishment.

Plant growth regulators (mg/l)	Variety	Days to response	% of meristems responded	Morphogenic response	
				Shoot	Root
KIN 0.1+GA_3 0.1	Lal Shill	5-6	70	+	+
	Patnai	5-7	68	+	+
	Shill Bilati	6-8	70	+	+
	Lal Pakri	7-8	72	+	+
	Sada Gutti	5-8	56	+	-
	Challisha	10-14	45	+	-
KIN 0.1+GA_3 0.5	Lal Shill	5-6	78	+	+
	Patnai	4-5	73	+	+
	Shill Bilati	5-7	56	+	+
	Lal Pakri	6-8	61	+	+
	Sada Gutti	5-7	56	+	+
	Challisha	6-8	61	+	+
KIN 0.5+GA_3 0.1	Lal Shill	5-7	72	+	+
	Patnai	6-7	61	+	+
	Shill Bilati	6-8	51	+	+
	Lal Pakri	6-8	56	+	+
	Sada Gutti	5-7	67	+	+
	Challisha	5-9	54	+	+
KIN 0.5+GA_3 0.5	Lal Shill	5-8	72	+	+
	Patnai	7-9	64	+	-
	Shill Bilati	6-7	53	+	+
	Lal Pakri	7-8	66	+	+
	Sada Gutti	7-9	50	+	+
	Challisha	9-10	44	+	+
LSD at 5% level			5.76		

+ = Positive responses, – = Negative responses.

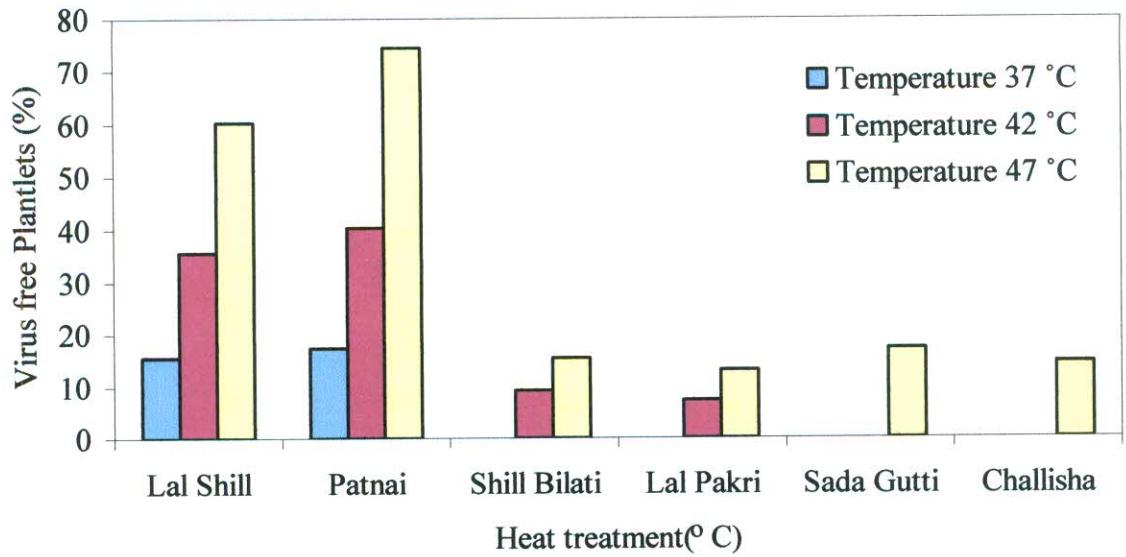


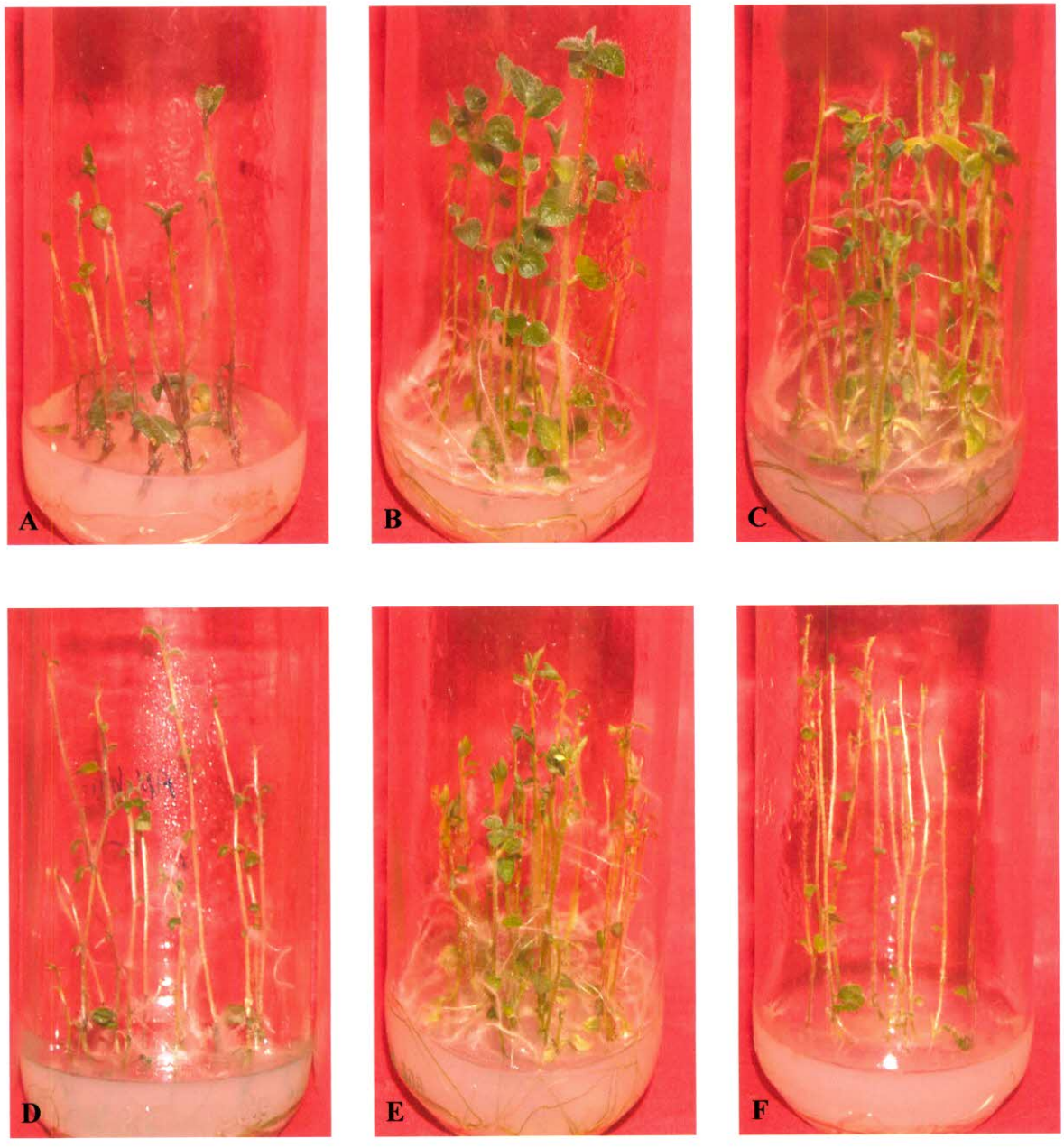
Figure 2.2: Effect of heat shock temperatures on the establishment of virus free meristem culture of six IPVs.

2.3.4. **Shoot Multiplication**

2.3.4.1. *Selection of growth regulator for maximum shoot proliferation*

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 in to MS semisolid basal medium supplemented with different types of growth regulators (Cytokinin, auxyn and gibberelic acid) either singly or in combination in order to find out suitable culture media for rapid shoot multiplication. Days required shooting initiation, percentage of explants showing shoot proliferation, number of shoots/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 the results are presented in **Table 2.3** and shown in **Plate 3.**

Plate 3



Figures: A-F: Multiplication of virus free plantlets from nodal segments on MS_0 medium of six IPVs: Lal Shill (A), Patnai (B), Shill Bilati (C), Lal Pakri (D), Sada Gutti (E) and Challisha (F).

Table 2.3. Effect of different concentrations and combinations of cytokinin, auxin and gibberellin in MS medium on shoot multiplication from meristem derived shoot of Lal Shill.

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 0.1 + 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 0.5	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

Shoot multiplication from established meristems were highly influenced by the type of cytoykinins with or 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 while 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/explant ranged from 1.5-3.5 and the length of the longest shoot ranged from 3.1-4.5 cm. However, the performance 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 or with an auxin or GA_3 and the percentage of shoot proliferation ranged from 47-85%, number of shoots ranged from 3.0-4.3 and length of shoot ranged from 6.1-9.6 cm. The cultured explants produced the highest number of shoots/explant (4.3) in medium containing 0.5 mg/l BAP + 0.5 mg/l GA_3 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 0.5 mg/l BAP + 0.5 mg/l KIN. Lowest 1.5 number of shoots/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. Lal Shill. 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/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.

2.3.4.2. *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/explant and length of shoot were recorded after 4 weeks of culture and the results are presented **Table 2.4.**

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 shoots/explant (3.3) and highest length of shoot (8.0 cm).

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 concentrations. Number of shoots/explants ranged from 4.3-4.7. Highest (4.7) number of shoots/explant was recorded in 30 g/l sucrose and lowest (4.3) number of shoots/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.

Table 2.4. Effect of carbon sources on shoot multiplication from nodal segments of meristem derived shoots of IPV Lal Shill in MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l GA_3.

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

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

In case of glucose percentage of shoot proliferation ranged from 62-70%, number of shoots/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/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/explant ranged from 2.0-2.3. The highest (2.3) number of shoots/explant was recorded in 30 g/l and lowest (2.0) in 20 g/l fructose. Length of shoot ranged from 4.6-5.8 cm. The highest length (5.8 cm) was found in 30 g/l and the lowest (4.6 cm) in 40 g/l fructose. Among the four carbon sources studied the highest (84.3%) percent of shoot proliferation, the highest (4.5) number of shoots/explant and the highest (8.5 cm) length of shoot were recorded in sucrose containing media and the lowest 65.0% of shoot proliferation was observed in local sugar. The lowest number of shoots (2.1) and the lowest length (5.1 cm) of shoot both were found in fructose containing media.

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

2.3.4.3. *Effect of genotypes and sucrose on shoot multiplication*

Six cultivars of potato viz. Lal Shill, Patnai, Shill Bilati, Lal Pakri, Sada Gutti and Challisha, and two concentrations of sucrose viz. 3% and 6% were used for this experiment. The meristem derived *in vitro* shoots were used as explants for this experiment. Nodal segments from these *in vitro* shoots of six 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/explant and shoot length were recorded after 4 weeks of culture and the results are presented in **Table 2.5.**

Table 2.5. Effect of genotypes and sucrose concentration on shoot multiplication

Treatments		% of explants developed shoot	No. of shoots/explant	Length of shoot (cm)
Sucrose (%)	Cultivar			
Sucrose 3%	Lal Shill	82	4.3	7.6
	Patnai	87	4.2	7.2
	Shill Bilati	83	2.0	8.8
	Lal Pakri	81	3.4	9.1
	Sada Gutti	84	3.9	7.5
	Challisha	85	4.0	8.1
Sucrose 6%	Lal Shill	57	3.3	4.5
	Patnai	52	3.1	5.3
	Shill Bilati	69	2.6	3.0
	Lal Pakri	61	2.1	4.1
	Sada Gutti	63	3.2	2.4
	Challisha	55	2.4	4.3
Effect of sucrose concentration*				
	3%	83.6 a	3.6 a	8.0 a
	6%	59.5 b	2.7 b	3.9 b
Effect of variety				
	Lal Shill	69.5 b	3.8 a	6.0 c
	Patnai	69.5 b	3.6 b	6.2 b
	Shill Bilati	76.0 a	2.3 d	5.9 c
	Lal Pakri	71.0 b	2.7 c	6.6 a
	Sada Gutti	73.5 a	3.5 b	5.4 c
	Challisha	70.0 b	3.2 b	6.2 b

In a column means followed by same letter (s) do not differ significantly according to LSD value at 5% level. * LSD test was done on transformed data.

In case of media containing 3% sucrose it was observed that percentage of shoot proliferation ranged from 81-87, number of shoots/explant ranged from 2.0-4.3 and shoot length ranged from 7.2-9.1 cm. The highest (87%) percentage of shoot proliferation was recorded in Patnai and the lowest (81%) in Lal Pakri. The highest (4.3) number of shoots/explant was observed in Lal Shill and the lowest (2.0) in Shill Bilati. Highest shoot length 9.1 cm. was found in Lal Pakri followed by Shill Bilati. The lowest shoot length (7.2) was recorded in Patnai.

In 6% sucrose containing media the percentage of shoot proliferation ranged from 52-69%, number of shoots/explant ranged from 2.1-3.3 and shoot length ranged were 3.0-5.3 cm. The highest (69%) percentage of shoot proliferation was recorded in Shill Bilati and lowest 52% in Patnai. The highest (3.3) number of shoots/explant was found in Lal Shill and lowest (2.1) in Lal Pakri. The highest (5.3 cm.) length of shoot was observed in Patnai and the lowest (2.4 cm.) in Sada Gutti.

Among the two concentrations of sucrose the highest (83.60%) percentage of shoot proliferation, highest (3.6) number of shoots/explant and highest (8.00 cm.) length of shoot were found in 3% sucrose containing media. On the other hand all of the characters were found the lowest in 6% sucrose.

Among the six potato cultivars highest (76%) percentage of shoot proliferation was found in Shill Bilati and the lowest (69.5%) in Lal Shill and Patnai. The highest (3.8) number of shoots/explant was recorded in Lal Shill and the lowest (2.3) in Shill Bilati. The highest (6.6 cm) length of shoot was observed in Lal Pakri and the lowest (4.9 cm) in Challisha. From this experiment it was concluded that 3% sucrose containing media was found to be better than 6% sucrose for shoot multiplication in potato.

2.3.4.4. *Effect of genotypes and number of explants/bottle on shoot multiplication*

For all six potato cultivars viz. Lal Shill, Patnai, Shill Bilati, Lal Pakri, Challisha, Sada Gutti and 6 and 8 node cuttings/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 explants/bottle on number of shoots/explant, number of branches/shoot and shoot length were recorded 4 weeks after culture and the results are presented in **Table 2.6.**

Table 2.6. Effect of genotypes and number of explants/bottle on shoot multiplication

Treatments		No. of branches/ shoot	No. of shoots/ explant	Length of shoot (cm)
No. of explants	Cultivar			
6 explants/bottle	Lal Shill	7.5	4.1	8.7
	Patnai	6.3	3.4	8.6
	Shill Bilati	5.8	3.5	7.8
	Lal Pakri	7.1	4.2	7.5
	Sada Gutti	6.2	3.6	7.6
	Challisha	7.3	4.0	8.5
8 explants/bottle	Lal Shill	3.5	3.5	7.1
	Patnai	3.1	3.0	6.7
	Shill Bilati	4.1	2.6	8.5
	Lal Pakri	3.6	2.1	5.5
	Sada Gutti	3.2	2.4	8.2
	Challisha	3.8	3.2	5.6
Effect of no. of xplants/bottle*				
6 explants/bottle		6.7 a	3.8 a	8.1 a
8 explants/bottle		3.5 b	2.8 b	6.9 b
Effect of variety				
Lal Shill		5.5 a	3.8 a	7.9 a
Patnai		4.7 a	3.2 a	7.6 a
Shill Bilati		4.9 a	3.0 a	8.1 a
Lal Pakri		5.3 a	3.1 a	6.5 b
Sada Gutti		4.7 a	3.0 a	7.9 a
Challisha		5.5 a	3.6 a	7.0 b

In a column means followed by same letter (s) do not differ significantly according to LSD value at 5% level. * LSD test was done on transformed data

In case of 6 explants/bottle the highest (4.2) number of shoots/explant was recorded for Lal Pakri and the lowest (3.4) in Patnai. The highest (7.5) number of branches/shoot was observed in Lal Shill and the lowest (5.8) in Shill Bilati. The highest length (8.7 cm.) of shoot was recorded in Lal Shill and the lowest (7.5 cm.) in Lal Pakri.

In case of 8 explants/bottle the highest number (3.5) of shoots/explant was recorded in Lal Shill and the lowest (2.1) in Lal Pakri. The highest number (4.1) of branches/shoot was found in Shill Bilati and the lowest (3.1) in Patnai. The highest (8.5 cm.) shoot length was recorded in Shill Bilati and the lowest (5.5 cm.) in Lal Pakri.

Of 6 and 8 explants/bottle the highest (3.8) number of shoots/explant, and the highest (8.1 cm.) shoot length and the highest number of branches/shoot were found in 6 explants/bottle. Number of shoots/explant, number of branches/shoot and shoot length was found lowest in 8 explants/bottle. From this experiment for all six cultivars, 6 explants/bottle produced more shoots, branches and shoot length than 8 explants/bottle.

2.3.5. **Field Performance of Meristem Culture Derived Plantlets**

The data for the field performance of *in vitro* produced plantlets were recorded on percentage of successful plantlets, days to tuber initiation, days to maturity, plant height, number of branches/plant, number of leaves/plant, leaf area/plant, tuber numbers/plant, tuber weight/plant, tuber dry matter content and tuber grade %. The results are presented in **Table 2.7** and **2.8** and in **Plate 4.**

Generally *in vitro* plantlets need special care and take some time for establishment in the field. From **Fig. 2.3** (**Appendix IV**) it was observed that the variety Patnai showed the highest percentage of plantlets that survived in the field and was closely followed by Lal Shill. The lowest survival rate was observed in the indigenous variety Lal Pakri.

The number of days required for tuber initiation varied with the variety (**Table 2.7**). Sada Gutti took the maximum number of days (35.75) which was statistically different than the other varieties. While Patnai needed the shortest time period (29.09) for tuber initiation.

Plate 4

Figures: A-F: Field establishment of meristem derived plantlets.

A: Plantlets of after 30 days of plantation.
B: Plantlets of after 40 days of plantation.
C: Plantlets of IPV Shill Bilati, after 45 days of plantation.
D: Sada Gutti, after 45 days of plantlet plantation.
E: After 70 days of plantlet plantation of IPV Lal Pakri with minitubers.
F: Plantlets of IPV Challisha, after 70 days of plantation with minitubers

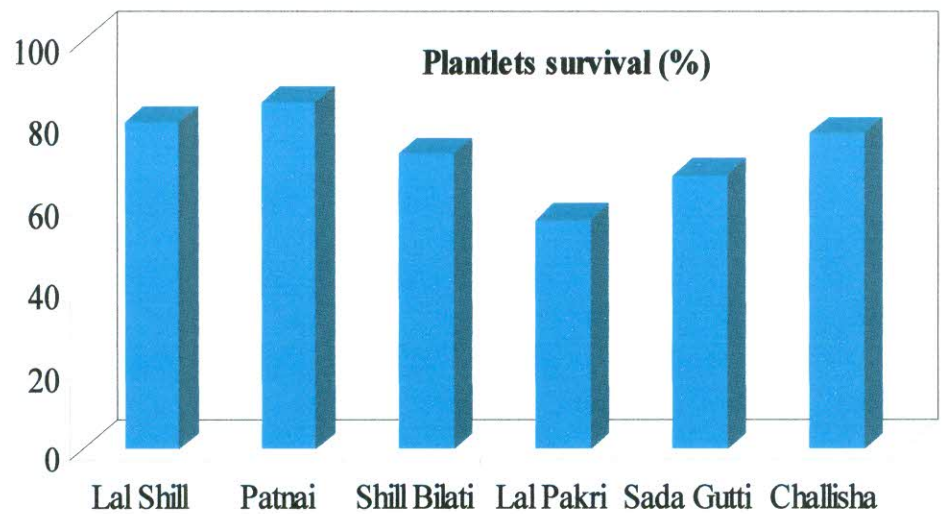


Figure 2.3. Survival percentage of *in vitro* produced plantlets of different IPVs in the field.

The maximum number of days (107.50) needed for the maturity of the plant was recorded in Sada Gutti that significantly differed from the rest of the varieties (**Table 2.7**). Regarding days to maturity Lal Pakri and Shill Bilati closely followed Sada Gutti, whereas the minimum number of days (89.60) required for the maturity of the plant was observed in the variety Patina followed by Lal Shill and they differed from each other statistically.

It was observed that the variety needing longer time for maturity attained comparatively more height except Challis and Lal Pakri (**Table 2.7**). Sada Gutti attained the highest height (85.62 cm) that took the maximum average number of days to reach maturity and its height was significantly different from the rest of the varieties. The minimum height (50.90 cm) was recorded in Lal Pakri, which was very closely followed by Challisha.

The highest number of branches/plant (10.20) was observed in Sada Gutti which was significantly higher than the rest of the varieties (**Table 2.7**). The remaining five varieties did not differ much from one another.

The number of leaves/plant varied with the variety (**Table 2.7**). Sada Gutti produced by far the highest number of leaves per plant (535.00) that differed significantly than all other

varieties and the rest of the varieties were statistically identical although the lowest number of leaves/plant (100.20) was observed in Patnai.

Sada Gutti produced the highest leaf area/plant (1755.11 cm^2) and this variety was followed by the two statistically identical varieties Patnai (600.12 cm^2) and Lal Shill (570.20 cm^2). The three other varieties produced the lowest leaf area/plant (**Table 2.7**).

Sada Gutti produced the highest number of tubers/plant (36.11). While the lowest number was recorded in Lal Shill (14.30) followed by Patnai (16.20) and they were statistically insignificant (**Table 2.7**).

The highest tuber weight/plant (128.70 g) was recorded in Patnai which was significantly higher than the rest of the varieties (**Table 2.7**). While the lowest weight of tubers/plant (24.40 g) was found in Challisha and was identical to Lal Pakri and Shill Bilati.

The highest percentage of dry matter (23.16) was produced by the variety Lal Shill followed by Patnai (22.51) and Sada Gutti (22.17) and they were statistically identical (**Table 2.7**). The lowest dry matter percentage (20.40) was recorded in the variety Challisha that was significantly difference in dry matter percentage was observed between Shill Bilati and Lal Pakri.

The results of the tuber grade percentage were presented in **Table 2.8**. From **Table 2.8** it was observed that the high yielding varieties, Patnai and Lal Shill, produced no tuber less than 10 mm size. Lal Pakri produced the highest (75.4%) percent of tubers in this grade and it was the lowest in Sada Gutti (64.5%). While significantly higher percentage of tubers of 10-20 mm size recorded in Lal Shill and Patnai than other indigenous varieties.

In 20-30 mm size, the highest percentage was observed in Patnai (78.4%) followed by Lal Shill (76.2%). While the lowest percentage was observed in Challisha (18.3%) followed by Lal Pakri (20.1%). The indigenous varieties produced no tuber greater than 30 mm size and only the high yielding varieties produced tuber of this grade. The highest percentage of tuber in this grade was observed in Patnai (7.92%) followed by Lal Shill (7.68%).

Table 2.7. Performance of virus free *in vitro* produced planting materials of different potato varieties in the field

Varieties	DTI	DM	PH	BP	LP	LA	TP	TW	DM
Lal Shill	29.85c	92.40c	66.22b	3.98b	110.50b	570.20b	16.20d	99.00b	23.16ab
Patnai	29.09d	89.60d	62.50b	4.20b	100.20b	600.12b	14.30e	128.7a	22.51bc
Shill Bilati	32.40b	103.10b	78.34a	3.92b	123.00b	412.80c	24.10b	28.40d	21.42c
Lal Pakri	34.20a	103.50b	50.90d	3.42b	130.00b	280.00d	21.30bc	25.10d	21.86c
Sada Gutti	35.75a	107.50a	85.62a	10.20a	535.00a	1755.11a	36.11a	54.00c	22.17bc
Challisha	31.78b	98.10c	51.05c	4.10b	126.20b	290.25d	17.10cd	24.40d	20.40d

Means followed by same letter (s) in column do not offer significantly at 5 % level.

DTI= Days to tuber initiation, DM= Days to maturity, PH= Plant height, BP= Branches/plant, LP = Leaves/plant, LA= Leaf area, TP= Tuber numbers/plant, TW= Tuber weight/plant, DM= Tuber drymatter.

Table 2.8. Tuber grade of different potato varieties obtained from *in vitro* produced plantlets in the field

Variety	Percentage of tuber grades			
	< 10 mm	10-20 mm	20-30 mm	> 30 mm
Lal Shill	0	76.20	16.12	7.68
Patnai	0	78.40	14.60	7.92
Shill Bilati	70.00	22.50	7.5	0
Lal Pakri	75.40	20.10	4.50	0
Sada Gutti	64.50	25.15	10.35	0
Challisha	65.20	18.30	16.50	0

2.4. DISCUSSION

The results of the virus elimination of potato varieties by heat treatment and merstim culture revealed that the meristems which developed into rooted *in vitro* plantlets were relatively higher in the exotic varieties than the indigenous varieties at almost all levels of heat treatment (except 24°C). It was also observed that regeneration of plantlets was variety dependent which corroborates with Mellor and Stace Smith (1969) and Zhuk (1978). The *in vitro* plantlets which were initially healthier and consequently more tolerant to heat, the regeneration of plantlets from excised meristems was better. It was observed initially that the exotic varieties were healthier than the indigenous varieties. The present finding are in agreement with those of Khanom (1984) and Hossain (1987) who recorded better survival rate of exotic varieties than the indigenous potatoes.

The lower percentage of virus free plantlets in the indigenous varieties than the exotic varieties might be that initially the indigenous varieties were badly infected with three to five viruses in a single plant. Moreover, these varieties were infected with viruses for long time, possibly establishing an intimate relation between viruses and potato plant. Alternatively, it was observed that initially the percentage of PVX and PVY infected plant was much higher in the indigenous varieties than the exotic ones, making virus elimination difficult in the indigenous varieties. Some workers (MacDonald, 1973; and Mellor and Stace-Smith, 1977) reported that elimination of PVX and PVY is difficult. Furthermore, the authors reported that elimination of PVY is more difficult than PVX which agrees with the present results.

The maximum production of virus free plants in all the varieties at 35°C incubation temperature indicates this temperature to be the most effective among the temperature regimes studied. The present findings are in agreement with those of Faccioli and Rubies Autonell (1982); Dhingra and Sangar (1983).

According to Quack (1966) there is competition between production of virus particles and cell production during cell division in meristematic tissue and the capacity for nucleic acid synthesis is utilized for cell production to the detriment of virus multiplication. Alternately, Quak (1977) postulated that the absence of vascular elements in the meristem is a possible

reason as why the virus concentration is so low in meristem. Mellor and Stace-Smith (1977) proposed that viral replication requires enzymes that are normally available to the cells near the meristematic dome and when small tips are excised their growth processes are temporarily disorganized and the enzymes that are required for one or more steps of viral replication become unavailable thus interrupting the production of infective virus. On the other hand, Ingram (1973) suggested that virus eradication may be either due to some inactivating factor produced by the explant or to the effect of some constituent of the culture medium on the virus. While Dhingra and Sangar (1983) mentioned that the preheat treatment results in rapid plant growth and consequent inability of the virus to multiply at equally fast rate and hence a lower concentration of the viruses in infected hosts, particularly in their apical meristems.

The proliferation of *in vitro* axillary shoots not stimulated by BA. Instead, shoot initiation was prolonged and callus growth with emerging shoots was produced. Shoots emerging from such callus may have originated denotatively from callus or from buried meristem. Adventitious shoots are not preferred if a high degree of genotypic stability is required (Lawrence, 1981).

The ineffectiveness of BA to induce axillary shoot proliferation does not indicate that cytokinin in general is ineffective. Another cytokinin or a mixture of cytokinins may give different results. BA has been very effective in inducing shoot proliferation of potato from callus tissue (Jarret *et al.* 1980; Lam, 1975; Marani and Pisi, 1977). Synergism between adenine and cytokinins has been found effective in inducing *in vitro* shoot proliferation of tobacco (Skoog and Miller, 1957) and Plumbogo (Gresshoff, 1978) from stem segments. The additive effect of different cytokinin in axillary buds of Solanaceae is unknown.

In general, cytokinin overcame the inhibitory effect of the terminal meristem on axillary bud growth (Sachs and Thiemann, 1964). The physiological basis as to why cytokinins are not effective in activating axillary shoot growth in potatoes is unknown, however several influences are apparent:

(1) Potatoes (like tomatoes) have a very weak apical dominance, thus the necessity for stimulation by exogenous control is less.

(2) Removal of the shoot tip of the explant eliminates the inhibiting effect of the apical meristem, thus further removing the need for exogenous stimulation.

(3) The shoot used for explants may have high levels of endogenous cytokinin. In such cases, addition of exogenous cytokinins can be expected to be inhibitory.

The toxic effect of an applied cytokinin (BA) on axillary shoot growth was demonstrated in *Solanum andigna* (Wang and Wareing, 1979; Woolley and Wareing, 1972). Woolley and Wareing (1972) treated single nodal cuttings of *Solanum andigena* with BA. They found that only pretreated (exhausted from endogenous cytokinin) nodal cuttings responded well to applied BA. Wang and Wareing (1979) treated non-pretreated nodal cuttings with different concentrations of BA. 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 included 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/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 workers also get satisfactory results in potato crops in BAP + GA_3 combinations (Kassanis, 1957; Siddique, 1991; Hussey and Stacey, 1981).

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 occurred 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 on 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.

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. Lal Pakri under optimum conditions produced 17 shoots/bottle after 4 week of subculture when 6 explants were cultured/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/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.* (1980) and Hussey and Stacey (1981).

Plant regeneration from tissue culture of potato has been successfully applied to breeding programmes by many workers. For many years tissue culture techniques have been applied to improve potato production by means of micro propagation, pathogen elimination. Potato is usually vegetatively propagated and very much susceptible to the number of viral diseases, which causes remarkable decrease in yield. However, meristem culture is one of the important methods to produced virus free stock plants (Wang and Hu, 1982). Whereas, micro propagation does not permit the micro clones to be free form viruses. The shoot 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. If this explant is carefully excised so as not to contaminate it with sap from more mature leaves or stem tissue and it is placed in culture tube a virus free plant can be established. Thermotherapy or heat treatment has proved to be very effective in virus elimination (Towill, 1981; De, 1992). Meristem culture in combination with heat treatment can be a useful technique to eliminate different viruses. The present investigation was therefore under taken to eliminate viruses by the application of meristem culture in combination with heat treatment.

In the present study it was closely observed that the *in vitro* produced plantlets having relatively thicker and harder stem at the time of transplanting had a higher survival rate than

the less thicker and harder one. The dry matter content of thicker and harder stemed plantlets was presumed to be higher that could withstand transplanting shock and out side environment more effectively, resulting relatively higher survival rate in the field. The high yielding varieties gave rise to thicker and harder *in vitro* plantlets and consequently higher survival rate was observed in the field than the indigenous varieties. Sada Gutti was statistically identical to the exotic varieties in the percentage of successful *in vitro* plantlets in the field, possibly due to the reason mentioned earlier.

Generally the indigenous varieties took relatively longer time for tuberization than the high yielding exotic varieties. This agrees nicely to Siddique (1989) who reported that longer time is needed for tuber initiation in the indigenous varieties than the exotic ones under the climatic condition of Bangladesh.

Comparatively longer time was needed for maturity of the indigenous varieties than the high yielding varieties which agrees with Siddique and Rashid (1990) who mentioned that the indigenous varieties have a long growth cycle than the modern varieties under the climatic condition of Bangladesh. Actually the time lag from sowing/planting to maturity is an inherent characteristic that varies from crop to crop and variety to variety of a crop.

The normal to moderated plant height of all the indigenous and exotic varieties under the present findings do not agree with Hosaain (1991) who reported luxuriant growth of the indigenous potato varieties, attaining up to about 2 m height after cleaning from viruses. The probable reasons for such disagreement between the results of Hossain and the present study might be (i) the indigenous potato varieties used for the two studies were different, (ii) the artificial growth media in which meristems and subsequently single node cuttings were cultured, also differed in the above two studies.

The highest number of branches/plant was observed in Sada Gutti which was significantly higher than the rest of the varieties. The remaining five varieties did not differ much from one another. This finding also did not agree with Hossain (1991) probably due to different sets of varieties and different compositions of the culture media used for the two experiments.

The number of leaves/plant varied with the variety Sada Gutti produced by far the highest number of leaves/plant that differed significantly than all other varieties and the rest of the varieties were statistically identical although the lowest number of leaves/plant was observed in Patnai. These results also different from the findings of Hossain (1991) probably due to difference in varieties and media compositions as mentioned earlier.

The highest leaf area in Sada Gutti was accrued mainly from the highest number of leaves/plant. Although Patnai and Lal Shill produced lower number of leaves than the other indigenous varieties, yet they produced higher leaf area/plant than those varieties due to their bigger leaves.

The indigenous potato varieties after cleaning from viruses developed numerous stolons with or without any tuber as reported by Hossain (1991) contradicted with the present finding probably because of using different sets of potato varieties and composition of the culture media.

It was also observed that the higher number of tubers per plant resulted smaller-sized tubers/plant and vice versa. The high yielding varieties produced higher weight of tubers/plant compared to the indigenous varieties. It is a common phenomenon that the indigenous varieties produce numerous smaller-sized tubers than the high yielding varieties.

The high yielding varieties, Patnai and Lal Shill, produced no tuber less than 10 mm size. Challisha produced the highest percentage of tubers in this grade and it was the lowest in Sada Gutti. While significantly higher percentage of tuber of 10-20 mm size recorded in the exotic ones than all the indigenous varieties. In 20-30 mm size, the highest percentage was observed in Patnai followed by Lal Shill. While the lowest percentage was observed in Lal Pakri followed by Challisha.

Some indigenous varieties produced only a few numbers of tubers greater than 30 mm size. The highest percentage of tuber in this grade was observed in Patnai followed by Lal Shill.

2.5. SUMMARY

With a view to producing virus free plantlets, six indigenous potato varieties were taken. The seven days old *in vitro* plantlets of these varieties were given three levels (37, 42 and 47°C) of heat treatment for a period that varied from three to seven weeks. Meristems were excised when the foliage showed severe chlorosis or after seven weeks whichever came sooner.

It was observed from the results that the percentage of regenerated plantlets was relatively higher in Patnai at all the levels of heat treatment. Moreover, the percentage of regenerated plantlets varied with the variety and level of heat treatment.

The highest percentage of virus free plantlets at different levels of heat treatment before the excision of meristems was recorded in Patnai followed by Lal Shill while the lowest percentage was observed in Sada Gutti. Moreover, the percentage of virus free plantlets was much higher in Lal Shill and Patnai at all levels of heat treatment than the other varieties. It was also observed that no virus free plantlet was produced in Shill Bilati, Lal Pakri, Sada Gutti and Challisha when heat treatment was applied at 37°C to the *in vitro* plantlets before the excision of meristems. Furthermore, Sada Gutti and Challisha failed to produce virus free plantlet at 42°C heat treatment. In all the varieties the percentage of virus free plantlets increased with the increase of incubation temperature applied to the *in vitro* plantlets before the excision of meristems and the highest percentage of virus free plantlets was recorded in all the varieties at 47°C incubation temperature of *in vitro* plantlets.

Established meristems were subcultured onto MS liquid basal medium supplemented with cytokinin, auxin and gibberellic acid either singly or in combination. Among 22 media formulation the suitable media composition of 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 0.1 mg/l BAP +0.1 mg/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 proliferation 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 proliferation. Among

the six cultivars of potato viz. Lal Shill, Patnai, Sill Bilati, Sada Gutti and Challisha higher frequency of shoot proliferation was observed in Lal Pakri.

It was observed from the field study of *in vitro* produced plantlets that virus free indigenous potato varieties, Shill Bilati and Lal Pakri produced plants of normal height and the Lal Shill and Patnai varieties were much superior to others. Moreover, all the indigenous varieties produced more but smaller tubers/plant than the non-tissue cultured planting materials.

Chapter III

PRODUCTION AND FIELD EVALUATION OF MICROTUBER

3.1. INTRODUCTION

The potato tuber is morphologically a modified stem with a shortened and broadened axis with rather poorly developed scale leaves (Slater, 1963). Tuber initiation and growth are the result of cell division and cell enlargement (Khuri, 1996; Plaisted, 1957). The first indication of tuber initiation is a thickening of the first internode behind the apical bud of the stolon (Plaisted, 1957). The continued growth of the tuber depends primarily on the expansion of internodes already present in the apical buds (Cutter, 1978). The size of the tubers depends on the number of internodes that make up the tuberous portion of the apical axis (Cutter, 1978).

Potato tuberization is not restricted to the 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 the same as other tubers. *In vitro* tubers can be sessile on the nodes of the stem (Catchpole and Hillman, 1969; Mes and Menge, 1954; Sakha *et al.* 2004) or can be axillary or terminally formed on new growing shoots (Hussey and Stacey, 1981; Palmer and Smith, 1969; Stallknecht, 1972; Wang and Hu, 1982). The sizes of in vitro produced tubers are usually one centimeter or less (Stallknecht and Fransworth, 1982a) but larger sized tubers have also been reported (Lawrence and Barker, 1963; Wang and Hu, 1982). For this reason we have termed *in vitro* produced tubers as 'microtubers'.

In vegetatively propagated crops like potato (*Solanum tuberosum* L.) once systematically infected with a viral disease, the pathogen can be passed form one vegetative generation to next. In potato contamination by a pathogen can severely reduce the total yield of the crop (Hoque *et al.* 1996). Since many tropical countries are not able to produce high quality seed tubers due to a lack of vector-free production area, the importation of certified seed and the resultant high costs, can become a major constraint to potato production (Wattimena *et al.* 1983).

Microtuber is an alternative end product of micropropagation, produced by allowing *in vitro* plantlets to grow under the tuber inducing conditions. In recent years, interest has developed in many countries in the induction of potato tubers under *in vitro* condition for disease free microtuber production and for germplasm conservation and distribution (Gopal *et al.* 2004). Use of micropropagated plantlets for production of tuber seeds in field shows many difficulties (laborious and high failure rates) in many tropical countries. *In vitro* tubers have been used to remove this problem. Microtuber could be harvested from *in vitro* conditions, stored, shipped and planted conveniently. Besides these, in general microtuber-derived plants produced higher yield than cutting derived plants both under screen house and field conditions (Hussey and Stacey, 1984; Stallknecht and Fransworth, 1982b; Wang and Hu, 1982; Alam *et al.* 2003).

In vitro tuberization is a complex physiological process regulated by many factors (Zakaria, *et al.* 2008); cytokinin and photoperiods are two major factors of this process. Cytokinins such as KIN and BA have shown to stimulate tuber initiation *in vitro* under high sucrose level and total darkness (Hoque *et al.* 1996; Hussey and Stacey, 1984; Wang and Hu, 1982; Garner and Blake, 1989; Palmer and Smith, 1970). Considerable effect of photoperiod on *in vitro* tuberization was also noticed by other workers (Hussey and Stacey, 1984; Pruski, *et al.* 2003a). For *in vitro* tuberization an inductive medium with high sucrose, KIN and growth retardants are required (Lawrence and Barker, 1963; Hussey and Stacey, 1984; Estrada *et al.* 1986; Escalante, 1998; Chandra *et al.* 1988; Al-momani *et al.* 2000). High sucrose levels are necessary for optimal microtuber production (Yu *et al.* 2000). Sucrose with KIN led to the pushing of the plantlets to produce microtubers (Wattimena *et al.* 1983; Pelacho and Mingo-Castel, 1991; Kefi *et al.* 2000). Besides chemicals (carbon and KIN) and genotypes, importance of length of photoperiod for *in vitro* tuberization was also reported and 8 h light was recommended (Gregory, 1956; Pelacho *et al.* 1993; Coleman and Coleman, 2000; Pruski *et al.* 2002).

In the present investigation nodal segments from *in vitro* multiplied shoots derived through meristem culture of four potato cultivars were used for induction of microtubers.

3.1.1. **Objectives**

i) Selection of appropriate culture media formulation for *in vitro* tuberization.

ii) Production, harvesting, store and dormancy breaking of microtubers

iii) Evaluation of microtubers under field condition.

3.2. MATERIALS AND METHODS

3.2.1. **Materials**

Disease free (cheeked through ELISA test) meristem-derived plantlets of four potato varieties viz. Lal Shill, Patnai, Shill Bilati and Lal Pakri were used as primary source of explants for *in vitro* tuberization. Nodal segments of these *in vitro* grown stock plants (2-8 cm length with 5-6 leaves) were used for microtuber production.

3.2.2. **Methods**

3.2.2.1. Formation of microtuber

To induce microtuber, the explants were cultured on agar (8.0 g/l phytagar, promega, UK) solidified MS (Murashige and Skoog, 1962) medium supplemented with different levels of sucrose as source of carbon and different concentrations of BA and KIN for cytokinin adjustment. The cultures were maintained at 0, 8, 24 hour photoperiods and were incubated at 22 ± 2°C for 8-12 weeks. Microtubers were harvested after required days (8-12 weeks) of culture and stored at 4°C.

3.2.2.2. *Dormancy of microtuber and transplantation*

After breaking the period of dormancy generally (8-12 weeks) when 100% microtubers were sprouted, the microtubers of 4 potato varieties were ready to transplant in the field.

3.2.2.3. *Field evaluation*

Field preparation, methods of transplantation and field maintenance, all were done in the same way as done for meristem and callus derived plantlets.

3.2.3. **Data Collection**

3.2.3.1. *Data collection for in vitro tuberization*

Data were collected using the following parameters and the methods of data collection are given below:

i) Weeks to microtuber induction.

ii) Percentage of *in vitro* tuberization.

iii) Number of microtubers/shoot: Number of microtubers was counted for each shoot after 12 weeks of culture and average microtuber number per culture was calculated.

iv) Weight of microtubers/shoot: Total weight of induced microtubers was measured after 12 weeks of culture on the individual shoot basis from five randomly selected cultures and mean weight was recorded.

3.2.3.2. *Data collection for in vitro grown microtubers under field condition*

i) Days to microtuber sprouting: The days were counted from microtuber harvest to sprouting.

ii) Days to microtuber initiating to sprouting.

iii) Days to emergence of shoots from microtubers.

iv) Duration of growth period: Growth period was calculated from transplanting to harvest.

v) Plant height at maturity.

vi) No. of branches/plant.

vii) No. of tubers/plant.

viii) Tuber weight/plant.

3.2.3.3. *Data analysis*

The data recorded for different observations were analyzed using the statistical methods described by Gomez and Gomez, 1984.

3.3. RESULTS

3.3.1. **The Effect of KIN and Photoperiod on *in vitro* Tuberization**

This part of study was consisted of 15-treatment combination of photoperiod and KIN. These were three levels of photoperiod (0, 8, 24 h) and 5 levels of KIN (2, 4, 6, 8, 10 mg/l), percentage of shoots induced microtuber, microtuber number per shoot and weight of microtubers per shoot were considered as parameters for evaluating this experiment and the results are given in **Table 3.1**. In this experiment only one cultivar, Patnai was used and data were recorded 12 weeks of subculture **(Plate 5)**.

Among the three levels of photoperiod studied highest percentage (63.6%) of shoots induced microtubers was recorded in continuous dark. Highest number (3.11) of microtubers per shoot and highest microtuber weight (185.72 mg) were found in continuous light (24 h).

Among the five different concentrations of KIN studied highest percentage (63. %) of shoots induced microtubers was recorded in medium with 8 mg/l KIN. The highest number of microtubers per shoot (3.19) was recorded in 8 mg/l KIN and highest weight (157.16 mg) of microtubers per shoot was observed in 10 mg/l KIN.

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

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

Table 3.1. Effect of different concentrations of KIN and photoperiods on microtuberization of IPVs.

Treatment		% of shoots induced microtubers	Microtuber numbers/shoot	Weight of microtuber/ shoot (mg)
Photoperiods (hour)	KIN (mg/l)			
0	2.0	40	1.65	66.5
(continuous dark)	4.0	52	1.75	70.2
	6.0	68	1.84	97.2
	8.0	78	3.00	105.5
	10.0	80	2.25	40.7
8 h light/day	2.0	30	1.00	165.5
	4.0	42	1.03	100.2
	6.0	44	1.95	105.8
	8.0	59	2.08	130.5
	10.0	46	2.15	190.8
24 h light/day (continuous light)	2.0	29	2.51	115.5
	4.0	38	2.56	144.4
	6.0	41	3.48	210.2
	8.0	52	3.60	218.5
	10.0	44	3.40	240.0
Effect of photoperiod				
0		63.60	2.10	76.02
8 h		44.2	1.82	138.56
24 h		46.8	3.11	185.72
LSD at 5% level		2.71	0.30	16.56
Effect of KIN				
2.0		33.0	1.72	115.83
4.0		44.0	1.78	104.93
6.0		51.0	2.42	137.73
8.0		63.0	3.19	151.50
10.0		56.66	2.60	157.16
LSD at 5% level		5.44	0.51	21.44

Plate 5

Figures: A-F: Different stages of *in vitro* microtuber development.

A. Tuber initiated in IPV Lal Shill in 8 mg/l KIN medium.
B. Tuber formation in IPV Patnai in 8 mg/l KIN medium.
C. Tuber initiation in plantlet at vegetative stage
D. Immature tubers of IPV Lal Pakri
E. Mature tubers of IPV Lal Shill.
F. Dormancy breaking with 8 mg/l KIN after 12 weeks of IPV Patnai.

3.3.2. **Effect of BA and KIN on *in vitro* Tuberization**

In this experiment six different concentrations of BA and KIN (2-12 mg/l) were used in order to find out suitable culture media for microtuber induction in potato cv. Patnai. Weeks required initiating microtuberization, percentage of *in vitro* tuberization, number of microtubers per shoot and weight of microtuber per shoot were considered as parameters for evaluating this experiment. Data were recorded after 12 weeks of subculture and the results are presented in **Table 3.2**.

In case of BA, cultured explants started to induce microtubers within 7-10 days. The percentage of *in vitro* tuberization ranged from 28-58%, number of microtubers per shoot ranged from 1.3-2.9 and weight of microtuber per shoot ranged from 91.2-109.3 mg. The highest (58%) percentage of explants showed *in vitro* tuberization was obtained in medium containing 12 mg/l BA followed by 52% in medium with 10 mg/l BA. Lowest (28%) *in vitro* tuberization was observed in 2 mg/l BA. Highest number of microtubers 2.9 was recorded in 6 mg/l BA followed by 2.7 numbers of microtubers in medium with 4 mg/l BA. The lowest 1.3 numbers of microtubers was found in 2 mg/l BA. Highest weight (109.3 mg) of microtuber per shoot was recorded in 10 mg/l BA and lowest weight (91.2 mg) of microtuber per shoot was observed in 2 mg/l BA.

In media with KIN it was observed that cultured explants induced microtubers within 6-9 days. The percentage of *in vitro* tuberization ranged from 34-68%. Highest 68% *in vitro* tuberization was observed in 10.0 mg/l KIN followed by 62% *in vitro* tuberization in 8 mg/l KIN. Lowest 34% *in vitro* tuberization was observed in 2 mg/l KIN.

Number of microtubers per shoot ranged from 1.6-3.8. The highest number of microtubers (3.8) was recorded in 10 mg/l KIN and lowest 1.6 numbers of microtubers was found in 2 mg/l KIN.

Weight of microtubers per shoot ranged from 114.2-202.7 mg. The highest weight (202.7 mg) of microtuber was found in 10 mg/l KIN and lowest (114.2 mg) weight of microtuber was found in 4 mg/l KIN. From this experiment it was observed that KIN was better than BA for microtuber induction and 10 mg/l KIN was most effective and most preferred concentration for microtuber induction.

Table 3.2. Effect of different concentration of BA and KIN in MS medium with 30 g/l sucrose under continuous dark on *in vitro* tuberization of IPV, Patnai.

Growth regulators (mg/l)	Weeks to microtuber induction	% of *in vitro* tuberization	No. of microtubers/shoot	Weight of microtuber/ shoot (mg)
BA 2	8-9	28	1.3	91.2
BA 4	8-9	30	2.7	100.4
BA 6	7-8	46	2.9	104.5
BA 8	7-8	48	2.4	105.2
BA 10	8-9	52	1.9	109.3
BA 12	9-10	58	1.4	102.5
Mean		43.66	2.1	102.18
KIN 2	8-9	34	1.6	130.5
KIN 4	7-8	42	2.8	114.2
KIN 6	7-8	57	2.9	188.5
KIN 8	6-7	62	3.2	200.0
KIN 10	8-9	68	3.8	202.7
KIN 12	8-9	42	2.3	192.2
Mean		50.83	2.76	171.35
LSD for KIN at 5%		5.67	0.67	19.04
LSD for concentration at 5%		8.06	0.89	27.54

3.3.3. **Effect of Sucrose and Genotypes on *in vitro* Tuberization**

In this experiment four cultivars of potato viz. Lal Shill, Patnai, Shill Bilati and Lal Pakri and seven concentrations of sucrose (20, 30, 40, 50, 60, 70, 80 g/l) were used. The meristem-derived *in vitro* shoots were used as explant source for this experiment and were subcultured in MS medium contained 8 mg/l KIN. Cultures were maintained under continuous light. Data were recorded after 12 weeks of subculture and the results are shown in **Table 3.3**. Different stages of microtuber development are shown in **Plate 5**.

In cv. Lal Shill it was observed that percentage of *in vitro* tuberization ranged from 36-75%. The highest 75% shoots induced microtuber was observed in medium containing 60 g/l sucrose followed by 72% in 70 g/l sucrose. Lowest 36% *in vitro* tuberization was recorded in medium containing 20 g/l sucrose. Number of microtubers/shoot ranged from 1.4-3.8. The highest number (3.8) was found in medium containing 60 g/l sucrose. Lowest (1.4) number of microtuber was observed in 20 g/l sucrose. The weight of microtuber/shoot ranged from 92-212 mg. The highest microtuber weight (212 mg) was observed in medium containing 70 g/l sucrose followed by (208.5 mg) in 60 g/l sucrose containing medium. Lowest microtuber weight (92 mg) was observed in 20 g/l sucrose containing medium.

In vitro tuberization of Patnai ranged from 37-76%. The highest 76% of *in vitro* tuberization was observed in medium containing 50 g/l sucrose. Lowest 37% was found in 20 mg/l sucrose. Number of microtubers/shoot ranged from 1.4-3.5. The highest number (3.5) of microtubers per shoot was recorded in 60 g/l sucrose containing medium. Lowest (1.4) number of microtubers was found in 20 g/l sucrose containing medium. The microtuber weight/shoot ranged from 92.3-210.7 mg. highest microtuber weight (210.7 mg) was recorded in 70 g/l sucrose containing medium and lowest (92.3 mg) microtuber weight was found in 20 g/l sucrose containing medium.

In Shill Bilati the percentage of shoots induced microtubers ranged from 35-75%. Highest percentage (75%) of shoots induced microtuber in 60 g/l sucrose containing medium, followed by 71% in 70 g/l sucrose containing medium. Lowest 35% shoots induced microtubers in medium containing 80 g/l sucrose. Number of microtubers per shoot ranged from 1.5-3.8. Highest microtuber number per shoot (3.8) was recorded in medium having 60

g/l sucrose and lowest microtuber number per shoot (1.5) was recorded in 20 g/l sucrose containing medium. Microtuber weight/shoot ranged from 88.5-209.5 mg was observed. Highest microtuber weight (209.5 mg) was found in 70 g/l sucrose containing medium and lowest was recorded (88.5 mg) in 20 g/l sucrose containing medium.

In cv. Lal Pakri percentage of *in vitro* tuberization ranged from 33-70%. Highest percentage (70%) of *in vitro* tuberization was observed in 60 g/l sucrose containing medium and lowest percentage (33%) of microtuber was found in 80 g/l sucrose containing medium.

Number of microtubers/shoot ranged from 1.2-3.8. Highest numbers (3.8) of microtubers was found in medium supplemented with 60 gm/l sucrose and lowest numbers (1.2) was found in medium containing 20 g/l sucrose. Microtuber weight per shoot ranged from 58.2-149.4 mg. highest microtuber (149.4 mg) weight was found in medium containing 60 g/l sucrose and lowest (58.2 mg) microtuber weight was found in 20 g/l sucrose containing medium.

Among the four cultivars average highest percentage (72.5%) of shoots induced microtubers was found in 60 g/l sucrose containing medium and lowest (39.25%) was found in 20 g/l sucrose containing medium. The highest average number (3.72) of microtubers/shoot was found in 60 g/l sucrose containing medium and lowest was recorded (1.37) in 20 g/l sucrose containing medium. The highest average microtuber weight/shoot (192.05 mg) was recorded in 70 g/l sucrose containing medium and lowest (82.75 mg) was found 20 g/l sucrose containing medium.

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 microtuber formation was observed. The media containing 60 g/l and 70 g/l sucrose were found to be the better among seven sucrose concentrations.

Table 3.3. Effect of different concentrations of sucrose and IPVs on *in vitro* tuberization in potato.

Sucrose concentration (g/l)	% of shoots induced microtuber					LSD at 5% level for
	Lal Shill	Patnai	Shill Bilati	Lal Pakri	Mean (%)	
20	36	37	40	44	39.25	Variety
30	53	50	46	48	49.25	(V) = 3.22
40	68	69	62	63	65.5	Sucrose conc.
50	70	76	68	68	70.5	(C) = 5.67
60	75	70	75	70	72.5	V×C = 8.88
70	72	74	71	65	70.5	
80	55	62	35	33	46.25	
Mean	61.28	62.57	56.71	55.85		
	Number of microtubers/shoot					
20	1.4	1.4	1.5	1.2	1.37	Variety
30	2.8	2.8	2.7	2.9	2.8	(V) = 0.18
40	3.2	3.0	3.3	2.8	3.07	Sucrose conc. (C) = 0.23
50	3.3	3.0	3.2	3.3	3.2	V×C = 0.32
60	3.8	3.5	3.8	3.8	3.72	
70	3.2	1.5	3.2	2.9	2.7	
80	2.0	2.7	1.9	1.3	1.97	
Mean	2.81	2.55	2.8	2.6		
	Microtuber weight (mg)/shoot					
20	92.0	92.3	88.5	58.2	82.75	Variety
30	98.3	96.2	93.2	79.2	91.72	(V) = 5.32
40	133.2	125.3	132.3	92.1	120.72	Sucrose conc.
50	184.1	182.0	180.4	104.5	162.75	(C) = 7.66
60	208.5	204.3	202.6	149.4	191.2	V×C = 9.59
70	212.0	210.7	209.5	136.0	192.05	
80	105.4	102.5	99.0	77.1	96.0	
Mean	147.64	144.75	143.64	99.5		

3.3.4. **Effect of KIN and Genotypes on *in vitro* Tuberization**

In this experiment four cultivars of potato viz. Lal Shill, Patnai, Shill Bilati and Lal Pakri and three concentrations of KIN (6, 8, 10 mg/l) were used. The meristem-derived *in vitro* shoots were used as explant. Cultures were maintained under continuous light. Data were recorded after 12 weeks of subculture and are shown in **Table 3.4**.

For Lal Shill percentage of *in vitro* tuberization ranged from 68-72%. Highest 72% was observed in medium containing 8.0 mg/l KIN followed by 70% in 10.0 mg/l KIN. Lowest 68% *in vitro* tuberization was observed in medium containing 6.0 mg/l KIN. Number of microbuters/shoot ranged from 2.8-3.58. Highest numbers (3.58) of microtubers were observed in 8.0 mg/l KIN and lowest (2.8) numbers of microtubers were observed in 6 mg/l KIN. Microtuber weight/shoot ranged from 185.5-200.6 mg. Highest microtuber weight (200.6 mg) was recorded in 8 mg/l KIN. Lowest microtuber weight (185.5 mg) was found in 6 mg/l KIN containing medium.

For cv. Patnai highest percentage (69%) of *in vitro* tuberization was observed in 8 mg/l KIN containing medium. Lowest 54% was recorded in 10 mg/l KIN. Highest number (3.99) of microtubers per shoot were found in 8 mg/l KIN containing medium and lowest (2.85) number of microtubers were found in 6 mg/l KIN. Highest microtuber weight (204.3 mg) was found in 10 mg/l KIN containing medium and lowest weight (189.2 mg) was recorded in medium containing 6.0 mg/l KIN.

For cv. Shill Bilati highest percentage (60%) of shoots induced microtuber was recorded in 8 mg/l KIN and lowest 40% of shoots induced microtuber was recorded in medium having 10 mg/l KIN. Highest number (2.78) of microtubers was found in 8 mg/l KIN fortified medium and lowest number (2.65) was recorded in medium with 10 mg/l KIN. Highest microtuber weight (180.5 mg) was found in 8 mg/l KIN containing medium and lowest weight (138.5 mg) of microtuber per shoot was found in 6.0 mg/l KIN fortified medium.

In Lal Pakri the highest percentage (60%) of microtuber was recorded in both 8 mg/l and 10 mg/l KIN and the lowest (56%) was recorded in 6 mg/l KIN supplemented media. Highest numbers (2.5) of microtubers per shoot were found in 6 mg/l KIN containing medium and lowest (2.25) number of microtuber was recorded in 8 mg/l KIN containing medium. The

highest microtuber weight (170.6 mg) was found in medium containing 8 mg/l KIN and lowest weight (115.4 mg) was recorded in medium having 6 mg/l KIN.

Among the four cultivars highest percentage (70%) of shoots induced microtubers was recorded in Lal Shill. Lowest (52%) *in vitro* tuberization was recorded in cv. Shill Bilati, Highest number (3.56) of microtubers per shoot was found in Patnai and lowest number (2.33) was recorded in Lal Pakri. Highest microtuber weight (197.96 mg) was recorded in cv. Patnai and lowest weight (142.06) was found in Lal Pakri.

Among three concentrations of KIN (6, 8, 10 mg/l) highest percentage (65.25%) of shoots induced microtuber was observed in medium supplemented with 8 mg/l KIN. Lowest percentage (56%) of shoots induced microtubers was found in 10 mg/l KIN containing medium. Highest number (3.15) of microtubers were found in 8 mg/l KIN added medium and lowest (2.7) number was recorded in medium containing 6 mg/l KIN. Highest microtuber weight (188.02 mg) was recorded in 8 mg/l KIN and lowest weight (157.02 mg) was recorded in 6 mg/l KIN fortified medium.

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

Table 3.4. Effect of different concentrations of KIN and cultivar on microtuber development.

Treatment		% of shoots induced microtubers	Microtuber number/shoot	Weight of microtuber/shoot (mg)
Cultivar	KIN (mg/l)			
Lal Shill	6.0	68	2.80	185.50
	8.0	72	3.58	200.60
	10.0	70	3.45	192.40
Patnai	6.0	61	2.85	189.2
	8.0	69	3.99	200.4
	10.0	54	3.84	204.3
Shill Bilati	6.0	56	2.67	138.5
	8.0	60	2.78	180.5
	10.0	40	2.65	166.5
Lal Pakri	6.0	56	2.50	115.4
	8.0	60	2.25	170.6
	10.0	60	2.26	140.2
Effect of variety				
Lal Shill		70	3.27	192.66
Patnai		61.33	3.56	197.96
Shill Bilati		52	2.7	161.83
Lal Pakri		58.66	2.33	142.06
LSD at 5% level		10.39	0.27	29.29
Effect of concentration				
6.0 mg/l		60.25	2.7	157.02
8.0 mg/l		65.25	3.15	188.02
10.0 mg/l		56.0	3.05	175.85
LSD at 5% level		9.99	0.19	28.76

3.3.5. **Dormancy of Microtuber**

3.3.5.1. *Dormant period of microtuber*

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

Little effect of cultivar and level of KIN were observed on the length of dormant period as measured from harvest to sprouting. Dormancy measure from tuber initiation to sprouting showed a pronounced effect of KIN (**Table 3.5**). The KIN level of 8 mg/l shortened the dormant period for all cultivars. Storage temperature 20 ± 2°C was used in all these experiments. Different stages of microtuer emergence are shown in **plate 5**.

3.3.5.2. *Rate of shoot emergence of microtuber*

The time of emergence of planted and sprouting microtubers averaged from 11 to 16 days (**Table 3.6**). At planting Lal Pakri had a more uniform and longer sprout (1-2 mm), whereas the sprout length of other cultivars was generally less than 1 mm. For this reasons Patnai and Lal Pakri had the earliest emergence with small variation in time of emergence (**Table 3.6**). For earlier and uniform emergence microtubers with sprout length of about 2 mm is recommended.

3.3.5.3. *Rate of growth of microtuber*

Ten nodes stage is an appropriate size of plant for transplanting in to the field. Most of the plants produced compound leaves observed after the formation of 17 nodes stage. The rate of microtuber plant growth from emergence to ten nodes stage took 17-22 days (**Table 3.7**). A shorter period (+ 17 days) was required for Lal Shill and Lal Pakri and a longer period (+ 22 days) required for Shill Bilati. In this stage, the plant had reached a height of 13.7-16 cm (**Table 3.7**).

Table 3.5. Effect of KIN concentrations on microtuber dormancy.

Treatment		Microtuber initiation to harvest (wks)	Harvest to sprouting (wks)	Microtuber initiating to sprouting (wks)
Cultivar	KIN (mg/l)			
Lal Shill	6.0	7	8	16
	8.0	8	7	14
	10.0	6	8	15
Patnai	6.0	11	7	17
	8.0	12	6	16
	10.0	7	7	15
Shill Bilati	6.0	7	9	16
	8.0	8	7	17
	10.0	8	7	14
Lal Pakri	6.0	7	9	17
	8.0	8	7	16
	10.0	7	8	16

Table 3.6. Date of sprout emergence from microtubers of four IPVs.

Cultivar	Days to emergence	
	Average	100%
Lal Shill	13 ± 0.81	25 ± 1.31
Patnai	11 ± 1.11	21 ± 1.70
Shill Bilati	15 ± 1.14	27 ± 7.31
Lal Pakri	16 ± 0.76	20 ± 1.73

Table 3.7. Duration of growth period of micropropagated plants from emergence to transplanting stage (10 node long shoot) and sprout height at transplanting stage.

Cultivar	Duration in days	Sprout height (cm)
Lal Shill	17 ± 0.88	14.1 ± 0.14
Patnai	18 ± 0.78	14.9 ± 0.33
Shill Bilati	22 ± 1.14	13.7 ± 0.67
Lal Pakri	17 ± 1.00	16.0 ± 0.17

3.3.6. **Field Performance of Microtuber derived Plants**

The field was prepared with garden soil, composed and sand (1:1:1) and moistened uniformly. After breaking dormancy the microtubers were sown taking special care not to damage the sprouted microtubers. After resuming the new growth of potato plants derived from microtubers on soil and having sufficient extension of growth and leaves development and at the time of harvest, data were collected from randomly selected 10 plants from each variety on plant height, number of branches/plant, number of leaves/plant, number of tubers/plant and tuber weight/plant and mean value and SE of mean are calculated and the results were presented in **Table 3.8**.

In this experiment it was observed that the plant height ranged from 47.5-62.4 cm. The highest plant height (62.4 cm) was recorded in Patnai and lowest (47.5 cm) was observed in Shill Bilati. There are no significant differences in plant height among four varieties.

The number of branches/plant ranged from 3.0-3.85. The highest number (3.85) of branches/plant was recorded in Lal Pakri and lowest (3.0) was found in Patnai. Number of branches/plant was almost similar in four potato varieties.

In case of number of leaves/plant, the results ranged from 85.6-107.42. The highest number (107.42) of leaves/plant was recorded in Lal Shill and lowest (85.6) was noted in Lal Pakri.

The number of tubers/plant ranged from 23.3-30.42. The highest number (30.42) of tubers/plant was recorded in Lal Shill and lowest number (23.3) was found in Shill Bilati.

Tuber weight/plant ranged from 98.5-200.1 g. The maximum tuber weight/ plant (200.1 g) was recorded in Patnai followed by Lal Shill (192.2 g). The minimum weight of tuber/plant (98.5 g) was found in Lal Pakri. Tuber weight/plant of Lal Pakri was significantly different from other varieties.

In this experiment it was observed that the varieties, Lal Shill and Patnai showed better performance in all morphological and yield characters except number of branches/plant which was maximum in Shill Bilati and Lal Pakri. The tuber weight/plant in Lal Pakri was extremely low as compared to other three varieties.

3.4. DISCUSSION

Nowadays, the production and use of potato microtuber is becoming popular in many countries (Zhijun, *et al.* 2005). It can be handled much like normal seed potatoes; needs very small space for storing; can be shipped conveniently and planted either by hand or automated (Hussey and Stacey, 1984; Tovar *et al.* 1985; Chandra *et al.* 1988 and McCrown and Joyce, 1991). Moreover, microtuber can be a starting material for minituber production (Coleman *et al.* 2001). Using minituber technologies provide yet another alternative to scale-up the number of high quality seed potatoes (Jones *et al.* 1988; and Joung 1993). In addition, since microtuber technologies potentially provide a much more rapid means of multiplying potatoes than the conventional slow process of bulking tuber numbers in the field or greenhouse, they may be ideal for introducing new varieties/genotypes into the production stream in a minimum time.

In vitro tuberization is a unique process to produce germ free clones of potato. Potato microtubers produced *in vitro* can be used as a source of germplasm for conservation (Gopal *et al.* 2004), transfer between countries and seed certification schemes. Photoperiod plays an important role in the tuberization process (Gregory, 1956; Pelacho *et al.* 1994; Seabrook *et al.* 1993) and should be optimized in order to enhance tuber size. International potato center developed rapid, cost effective methods that involve the addition of BA, CCC (Chlorocholinechloride) and sucrose to the liquid medium used for propagation (Tovar *et al.* 1985).

For microtuber production nodal segments of *in vitro* multiplied shoots derived through meristem culture of four cultivars were used in the present investigation. The explants were sub-cultured on MS medium with high concentration of BA, KIN and sucrose and were incubated in different photoperiods (0, 8, 24 h) at 22 ± 2°C. The results are discussed in following paragraphs with an attempt to justify them.

In this experiment results reveal that the acceleration of microtuber formation varied with concentration, types of phytohormone and genotypes. KIN is required only if tuberization occurs in the absence of light. The optimum KIN concentration for dark tuberization was 8 mg/l. A cytokinin requirement for *in vitro* tuberization under continuous dark has been

recognized by the research workers (Palmer and Smith, 1970; Pelacho, *et al.* 1994; Rafiq, *et al.* 2004) and concentration varied from 2 - 10 mg/l.

High tuberization 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 3-4 months of subculture, tubers were formed even though no growth regulator was added to the culture media. In continuous dark it was observed that increase the level of KIN also increased the percentage of tuberization, number of microtuber/shoot and weight of microtuber/shoot. These results are in agreement with the results of Palmer and Smith, 1970.

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

There are reports that *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 (Hussey and Stacey, 1984; Wang and Hu, 1982; Stallknecht, 1972). In the present experiment, it was clearly observed that phytohormonses, especially cytokinins are very useful for the acceleration of *in vitro* tuberization in potato. Requirement of cytokinin for *in vitro* tuberization has also been reported by several workers (Wattimena *et al.* 1983; Kefi *et al.* 2000; Pelacho, *et al.* 1994; Wang and Hu, 1985; Kanwal *et al.* 2006). In this investigation it was observed that KIN is most effective for microutber induction and production. This was supported by other workers (Wattimena *et al.* 1983; Hussey and Stacey, 1984; Ziv *et al.* 1998).

In conclusion, it may be recommended that for acceleration of microtuber induction and production use of KIN is most needed and in this finding, KIN 10 mg/l was most effective.

The photoperiod studies (**Table 3.1**) showed that longer photoperiod increased microtuberization in Patnai in all levels of KIN. With KIN, short light period showed better

response than continuous dark and 24 h photoperiods produced significantly more tubers than 0 and 8 hour photoperiods at all levels of KIN. The effect of photoperiod on *in vitro* tuberization was also noticed by Hussey and Stacey 1981; Janet *et al.* 1993 and Pruski *et al.* 2002 in their *in vitro* shoots of potato. They observed that microtubers were formed in cultures incubated in 16 and 24 h photoperiods after 3 - 4 months of subculture, on the other hand Wang and Hu (1982) reported that more tubers were produced under 8 hour photoperiods than 16 hour photoperiods. Regarding use of photoperiod, it depends upon the genotype used and objective of microtuber productions. For the production of high percentage of shoots induced microtubers continuous dark period is recommended whereas for production of high number with high weight of microtuber/shoot, use of long photoperiod is recommended.

The results obtained in the present investigation (**Table 3.3**) showed that there was no significant difference between 5, 6 and 7% sucrose but on 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% (Catchpole and Hillman, 1969; Lawrence and Barker, 1963; Obata-Sasamoto and Suzuki, 1979; Palmer and Smith, 1970; Stalknecht and Farnsworth, 1982b; Wang and Hu, 1982).

Increasing the sucrose concentration from 2% to 8% increased the earliness and percentage of *in vitro* tuberization (Khuri and Morby, 1995). 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.

The result also showed that in all cases, the lowest percentage of microtuber formation was observed at lowest sucrose concentration (20 g/l). The result further showed that the frequency of tuberization increased with the increased concentration of sucrose and 60-70 g/l was found optimum. The concentration of sucrose above this limit resulted decrease of tuberization. This might be due to effect of high osmoticum (Khuri and Morbi, 1995).

From the results obtained it can be concluded that medium 60 and 70 g/l sucrose concentration is the best for *in vitro* tuberization in potato. For potato cv. Patnai 60 g/l sucrose is recommended for high weight of microtuber induction.

The length of the dormant period not only depends on cultivar and KIN concentration but also on the method of measuring dormancy. The common methods of measuring dormancy in potato are from harvest to sprouting (Coleman and Coleman, 2000; Boguchi and Nelsion, 1980). However, Cho, *et al.* (1983) have measured dormancy from planting to tuber initiation to sprouting.

Patnai tended to have a shorter (6 wks) dormancy and Lal Shill, Shill Bilati and Lal Pakri a longer (7 wks). The media with 8 mg/l level of KIN shortened (7 wks) the dormant period for all cultivars. Boguchi and Nelsion (1980), using dormancy experiments with regular tuber found that Norland had a shorter dormancy (40 days) and Russet Burbank had longer dormancy.

The time of emergence of sprouting microtubers averaged from 11-16 days. The rate of emergence of sprouted tubers depends on sprout length (Emilson, 1949; Yiem, *et al.* 1990; Moorby and Milthorphy, 1975), soil moisture (Letnes, 1958), soil temperature (Moorby, 1978) and mineral nutrition (Yiem, *et al.* 1990; Moorby, 1978) and on cultivars.

The potato plants derived from microtubers of four varieties in field condition was similar to look as that of normal seed propagated plants. After a month of growth, they were not visually different between the varieties. The ability to produce leaves/plant branches/plant and plant height depended on the cultivar. Goodwin and Brown (1980) and Pruski *et al.*, 2003b also found similar result that there were no differences in number of leaves/plant and number of branches/plant among the cultivars. But earlier workers (Hougland and Akeley, 1959) reported that Russet Burbank produced more plants/hill than Red Pontiac and Katahdin. The larger mean tuber weight/plant in Patnai, Lal Shill and Shill Bilati as compared to Lal Pakri might be due to intertuber competition and genotypes.

3.5. SUMMARY

The present investigation was undertaken with a view to establish a standardized protocol for large scale *in vitro* propagation of disease free potato seeds through *in vitro* tuberization.

Microtubers were induced to meristem derived *in vitro* grown shoots in test tubes by increasing the concentration of BA, KIN and sucrose in MS medium and KIN showed better performance than BA for microtuber induction and among the six concentrations (2-12 mg/l) of KIN, 10 mg/l KIN was the most effective and more preferred concentration for microtuber induction. In continuous dark it was observed that increase the level of KIN also increased the percentage of tuberization and number and weight of microtuber/shoot. By increasing the concentration of sucrose increased the frequency of *in vitro* tuberization. The media containing 60 g/l was found to be the best among seven concentrations (20-80 g/l) of sucrose tested followed by 70 g/l 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 KIN for all four cultivars. With KIN short day photoperiod gave a response similar to continuous dark. The 24 h photoperiods produced significantly more microtubers than 0 (continuous dark) and 8 h photoperiods at all levels of KIN.

The measuring dormant period of microtubers from harvest to sprouting, the KIN level of 8 mg/l shortened the dormant period for all four cultivars. A period of 17 days was required for Lal Shill and Lal Pakri and longer period (+ 22 days) for Patnai and Shill Bilati.

Field evaluation results indicate that among four varieties higher tuber weight was found in IPVs Patnai, Lal Shill and Shill Bilati than Lal Pakri.

Chapter IV

INDUCTION OF SOMACLONAL VARIATION AND FIELD EVALUATION

4.1. INTRODUCTION

The techniques of plant cell and tissue culture have become more popular and useful methods in many temperate countries, which are being applied to solution of problem in many agriculture and forestry (Rao and Lee, 1986). Callus culture is also one of the ways of *in vitro* propagation. Callus tissue means an unorganized proliferative mass of cells produced from isolated plant cells, tissue or organs when grown aseptically on artificial nutrient medium in glass vials under controlled experimental conditions. Cell and tissue culture are being explored as innovative breeding method for the genetic modification and improvement of plants. These techniques included *in vitro* propagation of desirable genotypes through anther culture, callus culture, protoplast culture, protoplast fusion and transformation. Efficient plant regeneration methods are required for these techniques to be useful in crop improvement. The plant may regenerate through *de nove* organization of shoot and root meristem. Callus induction is the way to generate somaclonal variation. Callus induction and development involve a complex relationship among the explants used to initiate the callus, the composition of the medium and the environmental conditions during the cultural period. There are two ways of *in vitro* plant regeneration, somatic embryogenesis and organogenesis. Both these methods are possible to initiate plantlets from mature and immature embryos.

Induction of callus is one of the major steps of genetic transformation. When non dividing quiescent cells from differentiated tissue are grown on a nutrient medium that supports their proliferation, the cells first undergo certain changes to active meristematic state and forming undifferentiated callus tissue is termed de differentiation. A multicellular explant generally comprises cells of diverse types. As a result the callus derived from it would be heterogeneous with respect to the ability of its component cells to form a whole plant or plant organs which is called re-differentiation (Bhojwani and Razdan, 1983).

Bohorovja *et al.* (1995) demonstrated callus induction from immature embryos using MS (Murashige and Skoog, 1962) and N_6 media (Chu *et al.* 1975) supplemented with different concentration of 2, 4-D. Basal MS media supplemented with 2, 4-D are commonly used plant growth regulator in potato tissue culture.

Plant derived from tissue culture has been variously referred to as somaclones or calliclones or protoclones and the variations displayed by such plants are simply called somaclonal variation. Somaclonal variation commonly appears in plant after tissue culture involving a callus state (Shepard *et al.* 1980; Larkin and Scowcroft, 1981; Barwale and Widholm, 1987; Shamima *et al.* 2003). Various factors have been attributed to its origins; e.g. cytoplasmic or nuclear mutation, (Shepard *et al.* 1980), polyploidy or other chromosomal abnormalities *in vitro* or *in vivo* (Ahloowahlia, 1982; Orton, 1983) or possibly transposable elements (Larkin *et al.* 1984). It has been suggested that somaclonal variation may be a way of generating useful genetic variation and selection for desired traits could be performed *in vitro*. Somaclonal variation may be a powerful tool for the production of regenerates tolerant to environmental stresses (Larkin and Scowcroft, 1981).

The original ploidy level of the plant or plant origin from which the explant is taken, may play an important role in somaclonal variation. Somaclonal variation has been presented a lot of significant contribution to plant breeding. Somaclonal variation among regenerated plants from callus and protoplast culture has been suggested as a useful source of potential valuable germplasm for plant breeding. The major benefit of somaclonal variation is to create variation in adapted genotypes. Recognition of new genotypes at the whole plant level and their efficient exploitation would however be very useful breeding programme. For the improvement of potato crop Shepard *et al.* (1980) suggested that it would be more profitable to improve a popular variety selectively rather than to create new one.

The potato somaclones were also screened for both late and early blight resistance (De, 1992). The present "Russet Burbank" variety of potato is highly susceptible to both those diseases. Among more than 800 plants a range of variation to late blight (*Phytophthora infestans*) was found and 2% of the somaclones were also able to transmit the disease resistant character through subsequent tuber generations. In addition, several other disease

4.2. MATERIALS AND METHODS

4.2.1. **Materials**

Internodal and leaf segments of *in vitro* grown potato plants obtained through meristem culture of four potato varieties viz. Lal Shill, Patnai, Shill Bilati and Lal Pakri were used as explants for the initiation of callus.

4.2.2. **Methods**

4.2.2.1. *Callus induction*

To induce callus internodal and leaf segments were taken and cultured onto MS (Murashige and Skoog, 1962) medium supplemented with different PGR and incubated in dark at 25±2°C for 6-8 weeks. MS medium was supplemented with different concentrations of 2, 4-D and NAA with BA and sucrose (30 g/l) for callus induction. After 42-56 days of incubation in the dark, the callus induction frequency was determined. The induced calli were rated as hard, compact, friable and white, creamy, greenish brown, light green and light yellow in colour.

4.2.2.2. *Subculture of callus*

After callus induction from the explants, the calli were transferred on to the fresh medium for further proliferation and maintenance. The organogenic callus was selected for plant regeneration.

4.2.2.3. *Plant regeneration*

Plants were regenerated by transferring the selected calli on to MS semisolid medium supplemented with two kinds of cytokinins viz., BA and KIN in alone or in combination with NAA for shoot initiation and multiplication. The cultures were incubated at 25±2°C under white light for 16/8h light/dark conditions. After 4-5 weeks, differentiations as well as shoot formation were observed. The number of calli producing shoots and total number of shoots were counted for each treatment. The shoot from the selected callus was excised and transferred on to MS_0 (without PGR) medium for further growth. The plantlets from each of individual calli were further multiplied by node culture using MS_0 medium.

resistant varieties were recovered. Such variants are resistant to early blight (*Alternaria solani*) and to multiple races of *Phytophthora infestans* (De, 1992). Callus induction and regeneration occurs through culture of 12 weeks old potato tuber by using MS inorganic compounds, Nitsch and Nitsch's organic compound and different concentrations of auxin and cytokinin with CH (Bragdo-Aas, 1977). Using high concentration of Auxin led to the callus production only and cytokinin did not play any role in this respect, cytokinin alone produced shoot formation without callus (Edriss *et al.* 1996 and Alam *et al.* 2003). Nasrin *et al.* (2003) induced callus from nodal, internodal and leaf explants of potato with the help of different plant growth regulators.

In most of the published procedures, potato callus formation is promoted by maintenance of explants in the presence of both cytokinins and auxins, For example Cearly and Bolyard (1997) used 2.0 mg/l BA and 0.2 mg/l NAA for callus initiation from leaf tissues of *S. tuberosum* cv. Katahdin. Subsequently shoot generation normally requires withdrawal of auxin. Hansen *et al.* (1999) used an auxin-free shoot regeneration medium containing gibberellic acid and BA or zeatin, for leaf explants of potato cvs. Posmo, Folva and Oleva. Plant regeneration procedures for potato can be classified as either a one step method where a single culture medium is used for all phases or a two step method (Edwards *et al.* 1991; Hansen *et al.* 1999; Khatun *et al.* 2003) where callus is initiated on one medium (containing auxin) and shoot formation and development on another medium (containing cytokinin).

4.1.1. **Objectives**

From the above point of view the present investigation on callus culture and subsequent plant regeneration in potato was undertaken with the following objectives:

1. Standardization of suitable media composition for rapid callus induction from internodal and leaf segments of potato explants and their maintenance.
2. Establishment of suitable media composition for rapid plant regeneration from callus.
3. Acclimatization and establishment of plantlets in soil condition.
4. Field evaluation of the somaclones.

4.2.3. **Field Evaluation**

Field preparation, acclimatization, transplantation and field maintenance were done in the same way as done for meristem-derived plantlets.

4.2.4 **Determination of Starch Content of Potato**

The starch content of potato was determined by the Anthrone method as described in Laboratory Manual.

A potato sample of 5 gm was cut into small pieces and homogenized well with 20 ml of water. The homogenate was then filtered through double layer of muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly starch. Then it was kept overnight in cold; the precipitate was collected by centrifugation at 3000 rpm for 15 minutes. The precipitation was then dried over a steam bath. After that, 40 ml of 1 M HCl was added to the dried precipitate and heated to 70°C. It was transferred to a volumetric flask and diluted to 100 ml 1 M HCl. Then 2 ml of diluted solution was taken in another 100 ml volumetric flask and made up to the mark with 1 M HCl.

An aliquot of 1 ml of the extract was pipetted into test tubes and 4 ml of anthrone reagent was added to the solution of each tube and thoroughly mixed. Glass marbles were placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes, then removed and cooled. A reagent blank was prepared by taking 1 ml of anthrone reagent in a test tube and treated as before. The absorbance of the blue-green solution was measured at 680 nm in a UV/VIS spectrophotometer. The amount of starch present in the potato was calculated from standard curve of starch.

4.2.5. **Data Recording**

4.2.5.1. *Data recording for in vitro grown plants*

Data were collected using the following parameters and the methods for data collection are given below:

(i) For callus

a) Percentage of explants induced callus: Explants were cultured in 25×150 mm culture tubes containing media with different concentrations of plant growth regulators for callus induction. After required days of culture percentage of callus induction was calculated using the following formula.

$$\text{Percentage of callus induction} = \frac{\text{Numbr of explants induced callus}}{\text{Number of cultured explants}} \times 100$$

b) Qualitative assessment of callus: After few days of culture explants started to develop callus in the medium with proper growth regulators but colour of callus varied in respect of growth regulators supplements. For this reason, different symbols were used to denote the different colour of callus as given below:

Description of callus colour	Symbols	Description of callus colour	Symbols
Light green	LG	Green	G
Greenish brown	GB	Brown	B

The different degrees of callus growth were assessed using different symbols as given below:

Description of callus formation	Symbols
No. callus formation	-
Trace callus formation	+
Moderate callus formation	+ +
Massive callus formation	+ + +

(ii) For shoot induction

a) Percentage of calli induced to develop shoot: Data on different parameters from different treatments of shoot proliferation were recorded after required days of culture. The percentage of calli induced to develop adventitious shoots were calculated using the following formula:

$$\% \text{ of Calli induced shoots} = \frac{\text{Number of calli induced shoots}}{\text{Total no. of calli cultured}} \times 100$$

b) Number of shoots per callus: Number of shoots per callus was computed after required days of culture. Mean number of adventitious shoots per callus was calculated using following formula.

$$\overline{X} = \frac{\sum x_i}{N}$$

Where, $\overline{X}$ = Average number of shoots

$\sum$ = Summation

X_i = Total number of shoots

N = Number of observation

c) Length of the longest shoot: Length of the longest shoot was measured in cm scales for each explant after 30 days of inoculation. Average length of the longest shoot was calculated by using above-mentioned formula.

4.2.5.2. *Data recording for field grown plant*

Data on the following characters were recorded from ten randomly selected plants (somaclones) of each variety.

i) Plant height
ii) Number of leaves/plant
iii) Number of tubers/plant
iv) Tuber weight/plant
v) The amount of starch (gm per 100 gm of potato)

$$= \frac{\textit{Weight of starch obtained}}{\textit{Weight of the sample}} \times 100$$

Plant height and number of leaves/plant were recorded 60 days after plantation and number of tubers/plant and tuber weight/plant was recorded 90 days after plantation.

4.2.6. **Data Analysis**

The data recorded for different observations were analyzed using the statistical methods described by Gomez and Gomez, 1984.

4.3. RESULTS

The present investigation was carried out for indirect regeneration through callus culture of potato. The experiments were conducted with two different types of explants viz., internodal and leaf segments from four potato varieties. The objective of this investigation was to establish a protocol for the induction of callus, plant regeneration from callus and field establishment and evaluation of different somaclones of potato developed through callus culture. The results of this part of study are described under different heads.

4.3.1. **Callus Induction from Internodal Explant**

Internodal segments were cultured in MS medium supplemented with different concentrations of 2, 4-D and NAA alone or in combinations of NAA with BA. The callus induction was observed within eight weeks after inoculation. The results are presented in **Table 4.1** and **Plate 6**. The results obtained from this experiment are described under the following sub heads.

4.3.1.1. *Effect of different concentrations of 2, 4-D on callus induction*

Among all the doses of 2, 4-D, maximum 70% of cultured internodal explants of IPV Lal Shill were induce to develop callus in medium fortified with 3.0 mg/l 2, 4-D. The maximum degree of callus formation was noted with 2.5-3.5 mg/l 2, 4-D. The calli developed on these medium were light green, green and brown in colour. The second highest (60%) percentage of callus induction from this explant was noted with 2.5 mg/l and 3.5 mg/l 2, 4-D. The callus developed with these media was light green and brown in colour and the degree of callus formation was massive. The lowest percentage (20%) of callus induction was noted with 1.0 mg/l 2, 4-D. The calli developed in this media was brown in colour but the degree of callus formation was very little.

For IPV Patnai the highest 70% of callus induction was also recorded in media having 3.0 mg/l 2,4-D. The calli developed in this media was greenish brown in colour and degree of

callus formation was massive. The second highest 60% of callus induction from internodal explant was observed in media having 2.5 mg/l and 3.5 mg/l 2, 4-D. The calli developed in these media was brown and light green in colour and degree of callus formation was moderate. The lowest percentage (20%) of callus induction was noted with 1.0 mg/l and 4.0 mg/l 2, 4-D. The calli developed with these media formulations were light brown and brown in colour and degree of callus formation was trace amount.

The highest 70% of callus induction from internodal explant of Shill Bilati was observed with 3.0 mg/l 2, 4-D. The maximum degree of callus formation was also recorded with this medium. The calli developed in this medium was light green in colour. The second highest 60% explants induced callus proliferation was noted in medium having 3.5 mg/l 2, 4-D. The calli developed in this medium was brown in colour and the degree of callus formation was massive. The lowest 10% of explants showed callus induction in medium with 1.0 mg/l 2, 4-D. The calli developed in this medium was light brown in colour and the degree of callus formation was very little.

For Lal Pakri, no callus induction was recorded in media having 1.0 mg/l and 1.5 mg/l 2, 4-D. The highest 60% of callus induction was observed in medium fortified with 2.5 mg/l 2, 4-D. The calli developed in this medium was light brown in colour and the degree of callus formation was massive. The second highest 50% of the internodal segments showed callus induction with 3.5 mg/l 2, 4-D. The calli developed in this medium was light brown in colour and the degree of callus formation was massive. Callus induction was the lowest (20%) recorded in medium having 2.0 mg/l 2, 4-D. The calli developed with this medium formulation was light brown in colour.

4.3.1.2. *Effect of different concentrations of NAA on callus induction*

No callus induction was recorded for internodal segments of Lal Shill, Patnai and Shill Bilati in media with 1.0 mg/l, 3.5 mg/l and 4.0 mg/l NAA. In Lal Shill, the highest 30% explant showed callus induction in medium supplemented with 2.0 mg/l NAA. The callus developed in this medium was light yellow in colour and the degree of callus formation was trace. The lowest 10% explant induced callus proliferation with 3.0 mg/l NAA. The calli developed in this medium was off white in colour and the degree of callus formation was trace amount.

For Patnai, no callus induction was noted in media having 1.0 mg/l, 3.5 mg/l and 4.0 mg/l NAA. The highest 30% of the explants showed callus induction with 2.5 mg/l NAA. The calli developed in this medium was creamish white in colour and the degree of callus formation was trace. The lowest 12% explant showed callus induction in media having 1.5 mg/l and 3.0 mg/l NAA. The callus developed in these media was creamish white in colour and the degree of callus formation was trace.

The highest 20% internodal explants of Shill Bilati induce to develop callus when they were cultured onto medium supplemented with 2.5 mg/l NAA. The degree of callus formation in this medium was very little and callus colour was light brown. The lowest 8% explants showed callus induction with 3.0 mg/l NAA. The callus developed in these media was light brown in colour and the degree of callus formation was trace amount. Internodal explants of Shill Bilati failed to induce any callus in media supplemented with 1.0 mg/l, 3.5 mg/l and 4.0 mg/l NAA.

Internodal explants of Lal Pakri were also failed to induce callus in media having 3.5 mg/l and 4.0 mg/l NAA. The highest 28% of explants showed callus induction that was recorded in 2.0 mg/l NAA fortified medium. The calli developed in this medium was light brown in colour and the degree of callus development was trace. The lowest 6% explants showed callus induction with 3.0 mg/l NAA. The calli developed in this medium were creamish white in colour and the degree of callus formation was trace amount.

4.3.1.3. *Effect of NAA with BA on callus induction*

Internodal segments of four potato varieties were inoculated on MS medium containing different combinations and concentrations of NAA + BA **(Table 4.1).** In Lal Shill, the highest 50% explants showed callus induction onto the medium having 1.0 mg/l NAA + 1.0 mg/l BA. The calli developed in this medium was light green in colour and the degree of callus formation was massive. The callus proliferating explants was the lowest (10%) noted in medium with 1.5 mg/l NAA + 1.0 mg/l BA. The callus developed in this medium was creamish white in colour and the degree of callus formation was trace amount.

For Patnai the maximum 40% callus inducing explants was recorded in medium fortified with 1.0 mg/l NAA + 1.0 mg/l BA. The callus developed in this medium was light green in colour and the degree of callus formation was moderate. The lowest 10% callus inducing

explants were observed in media having 0.5 mg/l NAA + 1.0 mg/l BA and 1.5 mg/l NAA + 1.0 mg/l BA. Callus colour was light yellow and creamish white and the degree of callus formation were trace in both media formulations. Patnai failed to induce any callus with 0.5 mg/l NAA + 0.5 mg/l BA.

For Shill Bilati maximum 40% explants showed callus induction was recorded in medium having 1.0 mg/l NAA + 1.0 mg/l BA. The callus developed in this medium was light green in colour and the degree of callus formation was moderate. The lowest 10% callus inducing explants were noted in the media having 0.5 mg/l NAA + 0.5 mg/l BA and 1.5 mg/l NAA + 0.5 mg/l BA. The callus colour was creamish white and light green in colour and degree of callus formation was trace. No callus was developed in the medium having 1.5 mg/l NAA + 1.0 mg/l BA for Shill Bilati.

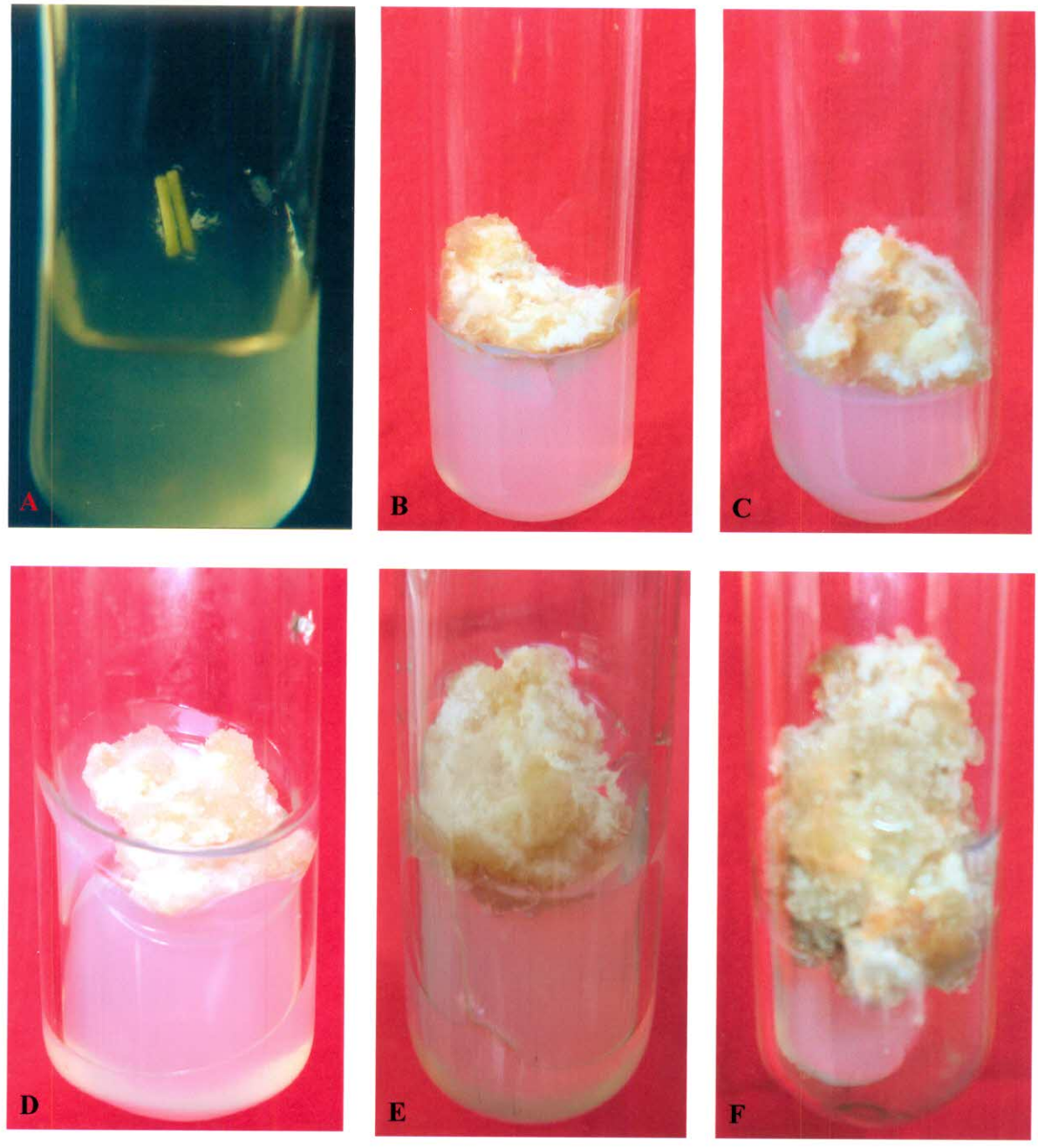
For Lal Pakri, the highest 40% callus inducing explants were recorded in media fortified with 1.0mg/l NAA + 1.0 mg/l BA. The callus developed in this medium was light green in colour and the degree of callus growth formation was moderate. The lowest 10% explants showed callus induction with 0.5 mg/l NAA + 1.0 mg/l BA and 1.5 mg/l NAA + 0.5 mg/l BA. The callus developed in these media was light green in colour and the degree of callus formation was trace. In media having 0.5 mg/l NAA + 0.5 mg/l BA and 1.5 mg/l NAA + 1.0 mg/l BA the explants did not produce any callus.

Table 4.1. Effect of different concentrations of 2,4-D or NAA singly or NAA in combination with BA in MS medium on callus induction from internodal explants of four cultivars of potato.

Plant growth regulators (mg/l)	Cultivars						Cultivars					
	Lalshil			Patnai			Shilbilati			Lalpakri		
	% of callus induction	Callus colour	Degree of callus formation	% of callus induction	Callus colour	Degree of callus formation	% of callus induction	Callus colour	Degree of callus formation	% of callus induction	Callus colour	Degree of callus formation
2,4-D												
1.0	20	B	+	20	LB	+	10	LB	+	-	-	-
1.5	30	B	+	40	LB	+	20	LB	+	-	-	-
2.0	40	LB	++	50	B	+	40	LB	+	20	LB	+
2.5	60	LG	+++	60	B	++	50	B	++	60	LB	+++
3.0	70	G	+++	70	GB	+++	70	LG	+++	40	B	++
3.5	60	B	+++	60	LG	++	60	B	+++	50	LB	+++
4.0	30	B	+	20	B	+	30	LB	++	30	LB	+
Mean	**44.28**			**45.71**			**40**			**28.57**		
NAA												
1.0	-	-	-	-	-	-	-	-	-	10	CW	+
1.5	20	CW	+	12	CW	+	10	LB	+	20	CW	+
2.0	30	LY	+	20	LY	+	10	LB	+	28	LB	+
2.5	20	CW	+	30	CW	+	20	LB	+	20	LB	+
3.0	10	CW	+	12	CW	+	8	LB	+	6	CW	+
3.5	-	-	-	-	-	-	-	-	-	-	-	-
4.0	-	-	-	-	-	-	-	-	-	-	-	-
Mean	**11.43**			**10.57**			**6.85**			**12**		
NAA + BA												
0.5 + 0.5	20	CW	+	-	-	-	10	CW	+	-	-	-
0.5 + 1.0	20	CW	+	10	LY	+	20	CW	+	10	LG	+
1.0 + 0.5	30	LG	++	30	LY	+	30	CW	+	20	LG	+
1.0 + 1.0	50	LG	+++	40	LG	++	40	LG	++	40	LG	++
1.5 + 0.5	30	CW	+	20	CW	+	10	LG	+	10	LG	+
1.5 + 1.0	10	CW	+	10	CW	+	-	-	-	-	-	-
Mean	**26.7**			**18.33**			**18.33**			**13.33**		

LB = Light brown, B = Brown, G = Green, LY= Light yellow, LG = Light green, CW = Creamish white, + = Trace callus, + + = Moderate, callus, + + + = Massive callus

Plate 6



Figures: A-F: Callus induction frtom internodal explant

A: Internode was placed 3.0 mg/l 2, 4-D medium for callus induction
B: Callus at early stage of proliferation in IPV Lal Shill after 8 weeks of inoculation on 3.0 mg/l 2, 4-D
C: Development of callus in IPV Patnai after 8 weeks of inoculation on 3.0 mg/l 2, 4-D
D: Compact callus formed in IPV Shill Bilati after 8 weeks of inoculation on 3.0 mg/l 2, 4-D
E: Development of callus in IPV Lal Pakri after 8 weeks of inoculation on 2.5 mg/l 2, 4-D
F: Friable callus in IPV Shill Bilati after 10 weeks of inoculation on 3.0 mg/l 2, 4-D

4.3.2. **Culture of Leaf Explants**

Leaf segments of four potato varieties were inoculated in MS medium supplemented with different concentrations of 2, 4-D and different combinations and concentrations of NAA and BA for callus induction. The results are presented in **Table 4.2.**

4.3.2.1. *Effect of different concentrations of 2, 4-D on callus induction from leaf explant*

For Lal Shill, the maximum 60% leaf explants were found to induce callus development with 2.5 mg/l 2, 4-D. The calli developed in this medium were light green in colour and the degree of callus formation was massive. The second highest percentage (50%) of callus induction was recorded in medium having 3.0 mg/l 2, 4-D. The calli developed in this medium were light brown in colour and the degree of callus formation was massive. The lowest 10% callus induction was observed in medium having 1.0 mg/l 2, 4-D. The calli developed in this medium were brown in colour and the degree of callus formation was trace amount.

In Patnai, the highest 40% leaf explants showed callus induction with 3.0 mg/l 2, 4-D. The calli developed in this medium were light green in colour and the degree of callus formation was moderate. The lowest 10% explants showed callus induction with 1.5 mg/l and 4.0 mg/l 2, 4-D. The callus developed in these media was brown in colour and the degree of callus formation was trace. The leaf explants of Patnai produced no callus in medium having 1.0 mg/l 2, 4-D.

In Shill Bilati, the highest 60% leaf explants showed callus induction in medium having 2.5 mg/l 2, 4-D. The calli developed in this medium were greenish brown in colour and the degree of callus formation was massive. The second highest 50% explants showed callus induction with 3.0 mg/l 2, 4-D. The calli developed in this medium were light brown in colour and the degree of callus formation was massive. The lowest 6% explants induced to develop callus in medium having 1.0 mg/l 2, 4-D. The colour of the callus was brown and degree of callus formation was trace amount.

The highest 40% explants of Lal Pakri showed callus induction in medium having 2.5 mg/l 2, 4-D. The calli developed in this medium were light brown in colour and the degree of callus formation was moderate. The lowest 8% callus formation was observed in medium

having 4.0 mg/l 2, 4-D. The calli developed in this medium were brown in colour and the degree of callus formation was trace. The leaf explants of Lal Pakri induced no callus in medium having 1.0 mg/l 2, 4-D.

4.3.2.2. *Effect of different concentrations and combinations of NAA with BA on callus induction from leaf explant*

For Lal Shill, the highest 50% leaf explants were induced to develop callus in medium having 1.0 mg/l NAA + 1.0 mg/l BA. The calli developed in this medium were light green in colour and the degree of callus formation was moderate. The second highest 40% explants showed callus induction with 1.0 mg/l NAA + 0.5 mg/l BA. The callus developed in this medium was light green in colour and the degree of callus formation was trace. The lowest 10% explants induced to develop callus with 0.5 mg/l NAA + 0.5 mg/l BA. The calli developed in this medium were brown in colour and the degree of callus formation was trace. No callus was observed in medium having 1.5 mg/l NAA + 1.0 mg/l BA.

The highest 30% callus induction from leaf explants of Patnai was recorded in media having 1.0 mg/l NAA + 1.0 mg/l BA and 1.5 mg/l NAA + 0.5 mg/l BA. The calli developed in these media were light green in colour and the degree of callus formation was trace. The lowest 10% callus induction was recorded in media having 0.5 mg/l NAA + 1.0 mg/l BA and 1.5 mg/l NAA + 1.0 mg/l BA. No callus was observed in the medium having 0.5 mg/l NAA + 0.5 mg/l BA.

For Shill Bilati, highest 50% of callus inducing leaf explants were recorded in medium having 1.0 mg/l NAA + 1.0 mg/l BA. The callus developed in this medium was light green in colour and the degree of callus formation was moderate. The second highest 40% explants showed callus induction in medium having 1.0 mg/l NAA + 0.5 mg/l BA. The calli developed in this medium were light green in colour and the degree of callus formation was moderate. The lowest 10% callus induction was observed in medium having 1.5 mg/l NAA + 1.0 mg/l BA. The callus colour was brown and the degree of callus formation was trace. The leaf explant of Shill Bilati produced no callus in medium having 0.5 mg/l NAA + 0.5 mg/l BA.

In Lal Pakri, the highest 30% callus induction was recorded in 1.0 mg/l NAA + 1.0 mg/l BA fortified medium. The calli developed in this medium were light green in colour and the degree of callus formation was trace. The lowest percentage (10%) of callus induction was noted in medium having 0.5 mg/l NAA + 1.0 mg/l BA. The calli developed in this medium were light green in colour and the degree of callus induction was trace amount. No callus was observed in media having 0.5 mg/l NAA + 0.5 mg/l BA and 1.5 mg/l NAA + 1.0 mg/l BA in Lalpakri.

4.3.3. **Shoot Regeneration from Callus**

This experiment was conducted to observe the effect of different concentrations and combinations of BA with NAA and KIN with NAA on shoot regeneration from calli derived from internode and leaf explants of different potato varieties. Data on percentage of calli induced to regenerate shoots and number of shoots/callus were calculated after required days of inoculation and length of longest shoot was also calculated 30 days after inoculation. The results are presented in **Table 4.3** and in **Plate 7** and **8**.

4.3.3.1. *Shoot regeneration from internode-derived callus*

The responses of internode derived calli are descrived under the following heads.

4.3.3.1.1. *Effect of BA + NAA on shoot regeneration from internode derived calli*

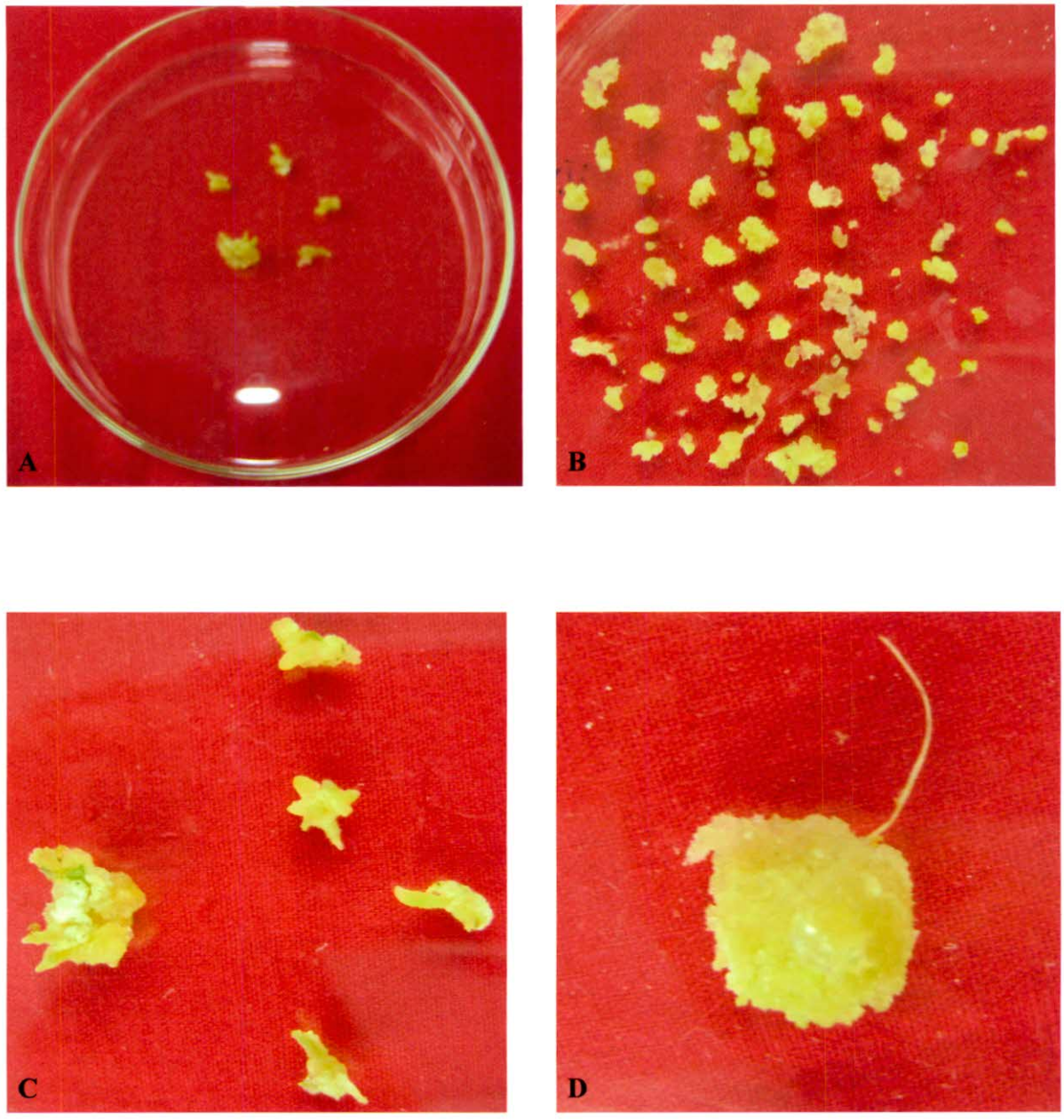
Calli derived from internodal explants of different potato varieties were sub-cultured on semisolid MS medium supplemented with different concentrations and combinations of BA with NAA. The results are shown in **Table 4.3**.

Table 4.2. Effect of different concentrations of 2,4-D and combinations of NAA with BA in MS medium on callus induction from leaf explants of different potato cultivars.

Plant growth regulators (mg/l)	Cultivars						Cultivars					
	Lalshil			Patnai			Shilbilati			Lalpakri		
	% of callus induction	Callus colour	Degree of callus formation	% of callus induction	Callus colour	Degree of callus formation	% of callus induction	Callus colour	Degree of callus formation	% of callus induction	Callus colour	Degree of callus formation
2,4-D												
1.0	10	B	+	-	-	-	6	B	+	-	-	-
1.5	20	B	+	10	B	+	20	B	+	10	B	+
2.0	30	B	+	20	B	+	30	B	+	20	B	+
2.5	60	LG	+ + +	30	LB	+	60	GB	+ + +	40	LB	+ +
3.0	50	LB	+ + +	40	LG	+ +	50	LB	+ + +	30	LB	+
3.5	40	LB	+	20	B	+	40	LB	+ +	20	B	+
4.0	20	B	+	10	B	+	20	B	+	8	B	+
Mean	**32.85**			**18.57**			**32.28**			**18.28**		
NAA + BA												
0.5 + 0.5	10	B	+	-	-	-	-	-	-	-	-	-
0.5 + 1.0	20	LB	+	10	LG	+	20	LB	+	10	LG	+
1.0 + 0.5	40	LG	+	20	LG	+	40	LG	+ +	20	LG	+
1.0 + 1.0	50	LG	+ +	30	LG	+	50	LG	+ +	30	LG	+
1.5 + 0.5	30	LG	+	30	LG	+	30	LG	+	20	LG	+
1.5 + 1.0	-	-	-	10	B	+	10	B	+	-	-	-
Mean	**25**	-	-	**16.7**	-	-	**25**	-	-	**13.33**	-	-

LB = Light Brown, G = Green, LG = Light green, B = Brown, LY = Light yellow, CW = Creamish white, + Trace callus, + + = Moderate callus, + + + = Massive callus

Plate 7

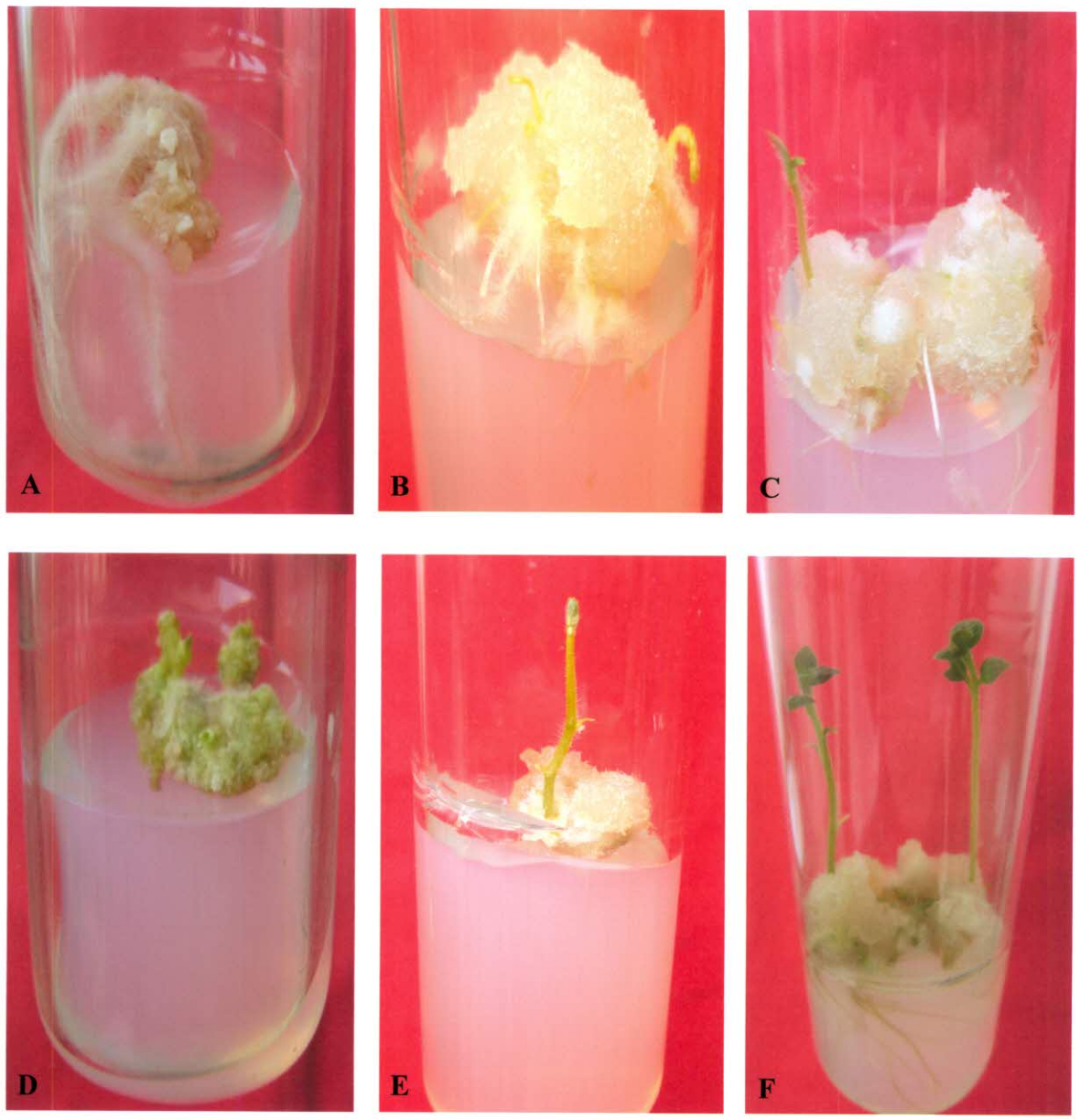


Figures: A-D: Shoot bud proliferation from internodal callus; the initial stage of plantlet development.

A-C: Shoot bud proliferation from leaf callus in MS medium supplimented with 3.1mg/l BA + 1.0 mg/l NAA in IPV Lal Shill.

D : A callus with numerous shoot bud in MS medium containing 3.0 mg/l BA + 0.1 mg/l NAA in IPV Shill Bilati.

Plate 8



Figures: A-F: Different morphogenic responses of calli derived from internodal explant.

A : Only roots were formed in IPV Shill Bilati at 3.0+1.5 mg/l BA + NAA mediam
B: Both roots and shoots were developed in IPV Shill Bilati at 3.0+1.0 mg/l BA + NAA
C: Both roots and shoots were developed in IPV Lal Pakri at 3.0+1.0 mg/l BA + NAA
D: The initial stage of shoot formation in IPV Lal Pakri at 3.0+0.1 mg/l BA + NAA
E: Only single shoot was developed in IPV Patnai at 3.0+1.0 mg/l BA + NAA
F: Both roots and shoots were formed in IPV Lal Shill at 3.0+1.0 mg/l BA + NAA

The highest 50% calli from internodal segment of Lal Shill induced to regenerate shoot in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The lowest 10% calli were recorded to developed shoot in medium containing 2.0 mg/l BA + 1.0 mg/l NAA. The highest number (5.5) of shoots/callus was observed in medium supplemented with 3.0 mg/l BA + 1.0 mg/l NAA. The lowest number of shoot was 2.1/callus recorded in medium having 3.0 mg/l BA + 0.5 mg/l NAA. The highest shoot length was recorded 2.2 cm in medium having 2.0 mg/l BA + 1.0 mg/l NAA. The lowest shoot length (1.8 cm) was observed after 30 days of inoculation in medium having 3.0 mg/l BA + 0.1 mg/l NAA.

For the internode-derived calli of Patnai, the highest 40% explants induced to develop shoots in media having 3.0 mg/l BA + 1.0 mg/l NAA and 3.0 mg/l BA + 1.5 mg/l NAA. The lowest 10% calli were recorded to induce shoot regeneration in media having 2.0 mg/l BA + 1.0 mg/l NAA and 2.0 mg/l BA + 1.5 mg/l NAA. The highest number of shoots (4.9) per callus was recorded in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The lowest number (1.4) of shoots/callus was observed in medium having 2.0 mg/l BA + 1.0 mg/l NAA. The longest shoot length was 3.1 cm recorded in medium supplemented with 2.0 mg/l BA + 1.5 mg/l NAA. The lowest shoot length 1.1 cm was recorded in medium having 3.0 mg/l BA + 0.5 mg/l NAA.

For the calli-derived from internodal segments of Shill Bilati, the highest 50% calli induced to develop shoots in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The lowest 11% calli were observed to induce regenerated shoots in medium having 2.0 mg/l BA + 1.0 mg/l NAA and 2.0 mg/l BA + 1.5 mg/l NAA. The highest number (5.3) of shoots/callus was recorded in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The lowest number of shoots/callus was 2.0 in medium having 3.0 mg/l BA + 0.5 mg/l NAA. The highest shoot length (2.3 cm) was recorded in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The lowest shoot length (1.6 cm) was observed in medium containing 3.0 mg/l BA + 0.1 mg/l NAA.

The highest 40% calli of Lal Pakri induced to develop shoots in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The lowest 6% calli were observed to induce shoot regeneration in medium having 2.0 mg/l BA + 1.0 mg/l NAA. The highest 4.7 shoots/callus was recorded in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The lowest number (1.2) of shoots/callus was

recorded in medium having 2.0 mg/l BA + 1.0 mg/l NAA. The highest shoot length (2.9 cm) was recorded in medium containing 2.0 mg/l BA + 1.5 mg/l NAA. The lowest shoot length (1.0 cm) was recorded in medium having 3.0 mg/l BA + 0.5 mg/l NAA.

4.3.3.1.2. *Effect of KIN with NAA on shoot regeneration*

The calli derived from internodal segments of different cultivars of potato were also subcultured onto MS semisolid medium supplemented with different concentrations and combinations of KIN with NAA for shoot regeneration (Table 4.3).

The highest 50% calli of Lal Shill induced to develop shoot regeneration in medium having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest 20% calli were observed to induce shoot regeneration in medium containing 3.0 mg/l KIN + 0.5 mg/l NAA. The highest number (6.5) of shoots/callus was observed in medium having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest number (2.3) of shoots/callus was observed in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest shoot length (5.2 cm) was observed in medium having 4.0 mg/l KIN + 0.5 mg/l NAA. The lowest shoot length (4 cm) was observed in medium having 3.0 mg/l KIN + 0.5 mg/l NAA.

The highest 50% calli Patnai induced to develop shoots in medium having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest 10% calli were recorded to induce shoot regeneration in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest number (6.9) of shoots/callus was recorded in medium supplemented with 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest number (2.3) of shoots/callus was noted in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest shoot length (6.2 cm) was observed in medium fortified with 4.0mg/l KIN + 1.0 mg/l NAA. The lowest shoot length (3cm) was observed in medium having 3.0 mg/l KIN + 0.1 mg/l NAA.

In Shill Bilati, the highest 50% calli were recorded to induce shoots in medium having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest 10% calli were recorded to induce shoot regeneration in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest number (6.3) of shoots/callus was observed in medium having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest number (2.1) of shoots/callus was recorded in medium supplemented with 3.0 mg/l KIN + 0.1 mg/l NAA. The highest shoot length (5.1 cm) was observed in medium having 4.0 mg/l KIN + 0.5 mg/l

NAA. The lowest shoot length (3.8 cm) was recorded in medium containing 3.0 mg/l KIN + 0.5 mg/l NAA.

The highest 50% internodal calli of Lal Pakri were observed to induce shoot regeneration in media having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest 10% calli induced to develop shoot regeneration in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest number (6.7) of shoots/callus was recorded in medium having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest number (2.1) of shoots/callus was observed in media having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest shoot length (6cm) was recorded in medium having 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest shoot length (2.8cm) was noted in medium having 3.0 mg/l KIN + 0.1 mg/l NAA.

4.3.3.2. *Shoot regeneration from leaf derived callus*

The results of shoot regeneration from leaf explants derived callus are shown in **Table 4.3**. Results obtained from these experiments are described under the following heads.

4.3.3.2.1. *Effect of BA + NAA on shoot regeneration from leaf-derived callus*

The highest 40% calli induced to develop shoot derived from leaf segments of Lal Shill in media having 3.0 mg/l BA + 0.1 mg/l NAA and 3.0 mg/l BA + 0.5 mg/l NAA. The lowest 15% calli was recorded to induce shoot regeneration in the medium supplemented with 2.0 mg/l BA + 1.0 mg/l NAA. The highest number (6.2) of shoots/callus was observed in medium having 3.0 mg/l BA + 0.1 mg/l NAA. The lowest number (2.2) of shoots/callus was recorded in medium supplemented with 3.0 mg/l BA + 1.5 mg/l NAA. The highest shoot length (2.5 cm) was observed in medium having 2.0 mg/l BA + 1.5 mg/l NAA. The lowest shoot length (1.3 cm) was observed in medium having 3.0 mg/l BA + 1.5 mg/l NAA.

In Patnai, the highest 50% calli induced to develop shoot regeneration in medium having 3.0 mg/l BA + 0.1 mg/l NAA. The lowest 12% calli was observed to induce shoot regeneration in medium having 2.0 mg/l BA + 1.0 mg/l NAA. The highest number (6.0) of shoots/callus was recorded in media having 3.0 mg/l BA + 0.1 mg/l NAA. The lowest number (3.6) of shoots/callus was noted in medium having 2.0 mg/l BA + 1.0 mg/l NAA. The highest shoot length (4.1cm) was recorded in medium supplemented with 2.0 mg/l BA + 1.0 mg/l NAA.

The lowest shoot length (1.1 cm) was recorded in medium having 3.0 mg/l BA + 1.0 mg/l NAA.

For leaf segments derived callus of Shill Bilati, the highest 50% calli induced to develop shoot regeneration in the medium having 3.0 mg/l BA + 0.1 mg/l NAA. The lowest 4% calli was recorded to induce shoot regeneration in medium having 3.0 mg/l BA + 1.5 mg/l NAA. The highest number (9.0) of shoots/callus was observed in the medium having 3.0 mg/l BA + 0.1 mg/l NAA. The lowest number (2.0) of shoots/callus was observed in medium containing 3.0 mg/l BA + 1.5 mg/l NAA. The highest shoot length (2.4cm) was observed in medium having 2.0 mg/l BA + 1.5 mg/l NAA. The lowest shoot length (1.1cm) was observed in 3.0 mg/l BA + 1.0 mg/l NAA and 3.0 mg/l BA + 1.5 mg/l NAA.

In Lal Pakri, the highest 40% calli induced to develop shoot regeneration in media having 3.0 mg/l BA + 0.1 mg/l NAA and 3.0 mg/l BA + 0.5 mg/l NAA. The lowest 3% callus was observed to induce shoot regeneration in media having 2.0 mg/l BA + 1.0 mg/l NAA and 3.0 mg/l BA + 1.5 mg/l NAA. The highest number (7.8) of shoots/callus was recorded in medium having 3.0 mg/l BA + 0.1 mg/l NAA and lowest number (3.4) was observed in medium having 2.0 mg/l BA + 1.0 mg/l NAA. The highest shoot length (3.9 cm) was observed in medium having 2.0 mg/l BA + 1.0 mg/l NAA and lowest shoot length (1 cm) was observed in medium having 3.0 mg/l BA + 0.5 mg/l NAA.

4.3.3.2.2. *Effect of KIN with NAA on shoot regeneration*

Calli derived from leaf explants of four potato cultivars were also sub-cultured on to MS semisolid medium supplemented with different concentrations and combinations of KIN and NAA for shoot regeneration and the results are presented in Table 4.3.

For leaf derived callus of Lal Shill, the highest 40% calli induced to shoot regeneration in medium having 4.0 mg/l KIN + 0.5 mg/l NAA. The lowest 5% calli were recorded to induced shoot regeneration in medium having 3.0 mg/l KIN + 0.5 mg/l NAA. The highest number of shoot per callus was recorded 5.3 when the medium was supplemented with 4.0 mg/l KIN + 0.5 mg/l NAA. The lowest number of shoot per callus was recorded 2.6 in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest shoot length (5.6 cm) was recorded in media having 4 mg/l KIN + 1.0 mg/l NAA and 4.0 mg/l KIN + 1.5 mg/l NAA.

The lowest shoot length (3.2 cm) was recorded in medium having 4.0 mg/l KIN + 0.5 mg/l NAA.

The highest 40% calli of leaf explants of Patnai induced to shoot regeneration in medium having 4.0 mg/l KIN + 0.5 mg/l NAA and lowest 6% were recorded to induce shoot regeneration in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest number of shoot per callus was recorded 6.0 in medium supplemented with 4.0 mg/l KIN + 1.0 mg/l NAA and lowest number of shoots per callus was recorded 2.6 in media having 3.0 mg/l KIN + 0.1 mg/l NAA and 3.0 mg/l KIN + 0.5 mg/l NAA. The highest length of shoot (4.6cm) was recorded in medium having 4.0 mg/l KIN + 1.0 mg/l NAA and the lowest shoot length (3.6 cm) was recorded in medium having 3.0 mg/l KIN + 0.1 mg/l NAA and 3.0 mg/l KIN + 0.5 mg/l NAA.

In Shill Bilati, the highest 40% calli induced to develop shoot regeneration in media having 4.0 mg/l NAA + 0.5 mg/l NAA and 4.0 mg/l KIN + 1.0 mg/l NAA. The lowest 2% calli were recorded to induce shoot regeneration in media having 3.0 mg/l KIN + 0.1 mg/l NAA and 3.0 mg/l KIN + 0.5 mg/l NAA. The highest number of shoots per callus was recorded 5.1 in medium having 4.0 mg/l KIN + 1.5 mg/l NAA and the lowest number of shoots per callus was recorded 2.4 in medium having 3.0 mg/l KIN + 0.1 mg/l NAA. The highest shoot length (5.4 cm) was recorded in medium having 4.0 mg/l KIN + 1.5 mg/l NAA and the lowest shoot length (3 cm) was observed in medium having 4.0 mg/l KIN + 0.5 mg/l NAA.

In Lal Pakri, the highest 40% calli were recorded to induce shoot regeneration in medium having 3.0 mg/l KIN + 1.0 mg/l NAA and the lowest 3% calli were recorded to induce shoot regeneration in media having 3.0 mg/l KIN + 0.1 mg/l NAA and 3.0 mg/l KIN + 0.5 mg/l NAA. The highest number of shoots per callus was recorded 6.0 in medium having 4.0 mg/l KIN + 1.0mg/l NAA and lowest number of shoots per callus was recorded 2.4 in media having 3.0 mg/l KIN + 0.1 mg/l NAA and 3.0 mg/l KIN + 0.5 mg/l NAA. The highest shoot length (4.5 cm) was recorded in medium having 4.0 mg/l KIN + 1.0 mg/l NAA and lowest shoot length (3.4 cm) was noted in media having 3.0 mg/l KIN + 0.1 mg/l NAA and 3.0 mg/l KIN + 0.5 mg/l NAA.

Table 4.3. Effect of different concentrations and combinations of BA with NAA and KIN with NAA in MS medium on shoot regeneration from internode and leaf explant-derived calli of different potato cultivars.

Plant growth regulators (mg/l)	Callus source	Cultivars						Cultivars					
		Lalshil			Patnai			Shilbilati			Lalpakri		
		% of calli regenerated shoots	No. of shoots/ callus	Shoot length (cm)	% of callus regenerated shoots	No. of shoots/ callus	Shoot length (cm)	% of calli regenerated shoots	No. of shoots/ callus	Shoot length (cm)	% of callus regenerated shoots	No. of shoots/ callus	Shoot length (cm)
BA+NAA													
2.0 + 1.0	Internode	10	2.4	2.2	10	1.4	2.6	11	2.1	2.0	6	1.2	2.4
	Leaf	15	4.4	2.3	12	3.6	4.1	20	4.2	2.1	3	3.4	3.9
2.0 + 1.5	Internode	20	3.1	2.1	10	1.9	3.1	11	3.0	2.0	20	1.7	2.9
	Leaf	20	4.1	2.5	20	4.1	3.7	20	3.9	2.4	20	3.9	3.5
3.0 + 0.1	Internode	30	2.6	1.8	20	1.6	2.2	20	2.4	1.6	20	1.4	2.0
	Leaf	40	6.2	1.6	50	6.0	1.4	50	9.0	1.4	40	7.8	1.2
3.0 + 0.5	Internode	30	2.1	2.0	30	2.6	1.1	30	2.0	1.8	30	2.4	1.0
	Leaf	40	5.1	1.5	30	5.8	1.2	40	4.9	1.3	40	5.6	1.0
3.0 + 1.0	Internode	50	5.5	2.1	40	4.9	2.1	50	5.3	2.3	40	4.7	2.0
	Leaf	30	3.1	1.4	20	5.1	1.1	30	2.9	1.1	20	5.0	1.9
3.0 + 1.5	Internode	40	2.9	2.1	40	2.1	2.1	40	2.7	2.0	30	2.0	2.0
	Leaf	20	2.2	1.3	30	5.1	1.6	4	2.0	1.1	3	5.0	1.5
Mean	**Internode**	**30**	**3.1**	**2.05**	**25.0**	**2.41**	**2.2**	**27.0**	**2.91**	**1.95**	**24.33**	**2.23**	**2.05**
	Leaf	**27.5**	**4.18**	**1.76**	**27.0**	**4.95**	**2.18**	**27.33**	**4.48**	**1.56**	**21.0**	**5.11**	**2.16**
KIN+ NAA													
3.0 + 0.1	Internode	30	2.3	4.3	10	2.3	3.0	10	2.1	4.1	10	2.1	2.8
	Leaf	20	2.6	4.1	6	2.6	3.6	2	2.4	3.9	3	2.4	3.4
3.0 + 0.5	Internode	20	3.7	4.0	20	5.1	3.2	30	3.5	3.8	20	5.0	3.0
	Leaf	5	3.1	3.6	20	2.6	3.6	2	2.9	3.4	3	2.4	3.4
3.0 + 1.0	Internode	30	3.1	4.4	20	4.1	5.3	30	3.0	4.2	20	3.9	5.1
	Leaf	20	3.1	4.1	20	3.1	4.1	20	2.9	4.0	40	3.0	4.0
4.0 + 0.5	Internode	30	4.2	5.2	40	5.6	5.3	20	4.0	5.1	40	5.4	5.1
	Leaf	40	5.3	3.2	40	3.6	4.1	40	5.0	3.0	30	3.4	3.9
4.0 + 1.0	Internode	50	6.5	4.7	50	6.9	6.2	50	6.3	4.5	50	6.7	6.0
	Leaf	30	4.7	5.6	30	6.0	4.6	40	4.5	3.4	30	6.0	4.5
4.0 + 1.5	Internode	40	4.4	5.1	40	6.0	4.6	40	4.2	5.0	40	6.0	4.5
	Leaf	30	5.1	5.6	20	4.1	4.2	30	5.1	5.4	20	3.9	4.0
Mean	**Internode**	**33.33**	**4.03**	**4.61**	**30**	**5.0**	**4.6**	**30**	**3.85**	**4.45**	**30**	**4.85**	**4.41**
	Leaf	**24.16**	**3.98**	**4.36**	**22.66**	**3.66**	**4.03**	**22.33**	**3.8**	**3.85**	**21.0**	**3.51**	**3.86**

4.3.4. **Field Evaluation of Callus Derived Plantlets**

The calli which were induced to develop shoots were isolated and sub-cultured on to agar solidified MS_0 (without growth regulator) medium for further shoot elongation and root formation. Well-developed shoots from ten-selected calli for each of the cultivars were excised and cultured separately and individually. Accession of somaclones were named and maintained as D1-D10, C1-C10, SB1-SB10 and LP1-LP10 for Lal Shill, Patnai, Shill Bilati and Lal Pakri, respectively. The plantlets for each callus accession were multiplied through internode cutting. These plantlets grown inside the test tubes were brought out from the growth chamber. After deplugging the culture tubes were kept in the room temperature for 4-5 days to bring them in contact to normal temperature. After 3-5 days of hardening the plantlets were taken out from test tube carefully. The acclimatized plantlets were transplanted on to the specially prepared field in complete randomized experimental design with three replications. There were total 600 plantlets per cultivar, 60 plantlets from each of the callus (20 plantlets/replication) were planted. Data were collected from 10 randomly selected plants of each somaclonal line of each replication and mean value was calculated. For the evaluation of somaclonal variations among the somaclones, plant height, number of leaves/plant, number of tubers/plant and tuber weight/ plant were used as parameter. Results are presented in **Tables 4.4-4.7** for Lal Shill, Patnai, Shill Bilati and Lal Pakri, respectively.

Considerable variation was noticed among the plants of the 10 callus lines (**Table 4.4**) of Lal Shill for all the characters whereas, variation among the plants of same somaclones was less pronounced. Among the different somaclones D7 showed highest plant height (124.16 cm) and lowest plant height (83.58 cm) was recorded in D2 callus line. LSD value reveals that the variation observed for plant height among some of the callus lines were statistically significant. The highest number of leaves/plant was recorded (26.88) for the plants of D4 callus line and lowest (15.26) for the plants of D5. Similarly the highest number of tubers/plant (24.34) was recorded for the plants belonged to callus line D3 and lowest number (11.65) was recorded for D8 callus line. Tuber weight/plant (351.46 g) was recorded the highest for callus line D3 and the lowest (156.32g) for the plants under the callus line D2. Analysis of variance followed by subsequent LSD test for these attributes supported real difference among the different callus lines. In addition to these, changes or modifications in some other characters were also observed. Plants with more green leaves, deformed leaves,

dwarfism and modification in canopy structure were also noted in some of the plants. Starch content of the somaclones ranged from 15.29-21.47. Maximum starch was recovered from D7 followed by D10 and D1, however, the variation among these three somaclones was not significant. The lowest starch was noted from D8.

Somaclonal variations among different callus lines of Patnai were also observed for all characters under study (**Table 4.5**). Plants developed from the callus line C8 displayed the highest (123.38 cm) plant height, highest number of leaves (52.78) per plant and highest tuber weight (337.75 g) per plant. Whereas, highest number (25.8) of tubers/plant was recorded for the plants grown from the callus line C4. On the other hand, the lowest plant height (82.58 cm) was observed in C2 and lowest number of leaves/plants (16.32) and lowest tubers weight/plant (123.8 g) was recorded in C1 and lowest number of tuber/plant (11.41) in C6 callus lines. Statistical analysis of the individual characters supported the existence of significant variation among the different callus lines. Starch content of the somaclones ranged from 15.61-21.01. Maximum starch was recovered from C10 followed by C3, while, the variation among these two somaclones was not significant. The lowest starch was noted from C10.

Remarkable variation was recorded among the plants of the 10 callus lines of cv. Shill Bilati (**Table 4.6**). Plants developed from the callus line SB3 displayed the highest plant height (126.5 cm), highest number of leaves/plant (33.5) and highest tuber weight/plant (272.10 g), and highest number of tubers/plant was recorded 46.72 in SB1 callus line. On the other hand, the lowest plant height (105.25 cm), lowest number of leaves/plant (18.3), lowest number of tubers/plant (27.3) and lowest tuber weight/plant (85.5 g) were recorded for the plants belonging to the callus lines SB10, SB8, SB5 and SB2, respectively. Statistical analysis of the individual character also supported the presence of significant variations. The maximum starch of the somaclones was obtained from SB5 followed by SB9 and SB3 while the variation among these three somaclones was not significant. The lowest starch content was noted from SB4.

In Lal Pakri variations among different callus lines were observed for all the characters under study (**Table 4.7**). Plants developed from the callus line LP3 displayed the highest plant height (142.82 cm), highest number of leaves/plant (35.54), highest tuber weight/plant (204.36 g) and

highest number of tubers/plant was recorded 68.39 in LP1 callus line. On the other hand the lowest plant height (106.34 cm) and lowest tuber weight/plant (89.65 g) was recorded in LP10 and lowest number of leaves/plant (20.36) and lowest number of tubers/plant (29.34) were recorded for the plant belonging to the callus lines LP8 and LP5, respectively. Starch content of the somaclones ranged from 14.95-19.51. The maximum starch was recovered from LP4 followed by LP3 and LP9. However, the variations among these three somaclones were not significant. The lowest starch content was noted from LP6.

Statistical analysis of the individual character also supported the existence of significant variation observed among the different callus lines. Like Lal Shill modification in other morphological characters were noted among the somaclones regenerated from the calli of Patina, Shill Balati and Lal Pakri.

Table 4.4. Somaclonal variation among randomly selected 10 somaclones of IPV Lal Shill.

Plant accession	Plant height (cm)	Number of leaves/plant	Number of tubers/plant	Tuber weight/ plant (g)	Starch content%
LalShill(basic)	103.54	20.92	16.20	199.00	18.06
D1	92.35	18.34	14.67	178.80	20.45
D2	83.58	18.45	12.56	156.32	19.02
D3	86.45	19.55	24.34	351.46	18.75
D4	114.26	26.88	12.80	163.35	19.35
D5	85.84	15.26	16.66	208.65	17.41
D6	108.36	19.48	16.45	282.76	18.23
D7	124.16	25.75	14.22	175.56	21.47
D8	122.38	24.78	11.65	336.25	15.29
D9	102.67	18.64	16.36	218.49	18.74
D10	105.45	17.22	14.34	190.78	21.42
Mean	102.64	20.48	15.47	223.76	18.92
LSD at 5% level	16.25	2.28	3.78	69.35	6.43

Table 4.5. Somaclonal variation among randomly selected 10 somaclones of IPV Patnai.

Plant accession	Plant height (cm)	Number of leaves/plant	Number of tubers/plant	Tuber weight/ plant (g)	Starch content%
Patnai (basic)	62.50	100.20	14.30	128.7	17.56
C1	91.33	16.32	14.69	123.80	17.95
C2	82.58	23.41	16.56	152.32	17.72
C3	84.41	19.55	12.31	165.46	19.24
C4	112.21	25.29	25.80	258.36	15.61
C5	82.74	19.21	18.66	204.61	19.07
C6	97.36	16.48	11.41	157.01	18.70
C7	111.16	24.89	13.22	267.75	17.55
C8	123.38	52.78	11.65	337.75	17.13
C9	99.67	19.21	17.38	217.41	18.31
C10	97.44	17.21	14.31	172.41	20.01
Mean	98.22	23.43	15.59	198.68	18.08
LSD at 5% level	15.48	3.21	2.14	60.43	5.67

Table 4.6. Somaclonal variation among randomly selected 10 somaclones of IPV Shill Bilati.

Plant accession	Plant height (cm)	Number of leaves/plant	Number of tubers/plant	Tuber weight/ plant (g)	Starch content%
ShillBilati(basic)	126.34	27.00	38.30	178.40	16.71
SB1	120.75	23.50	46.72	260.30	18.24
SB 2	114.30	22.58	28.65	85.50	17.49
SB 3	126.50	33.50	46.30	272.10	18.89
SB 4	106.35	22.85	34.45	175.50	15.37
SB 5	113.50	21.88	27.30	115.25	19.86
SB 6	123.40	26.40	32.50	160.75	16.28
SB 7	121.30	20.75	34.55	195.40	16.90
SB 8	116.40	18.30	46.25	210.25	17.21
SB 9	121.50	24.20	32.24	160.75	18.95
SB 10	105.25	27.30	27.35	120.92	18.25
Mean	117.78	24.38	35.87	175.92	17.65
LSD at 5% level	11.59	2.33	4.59	66.67	7.47

Table 4.7. Somaclonal variation among randomly selected 10 somaclones of IPV Lal Pakri.

Plant accession	Plant height (cm)	Number of leaves/plant	Number of tubers/plant	Tuber weight/ plant (g)	Starch content%
LalPakri(basic)	130.90	30.00	40.30	175.10	17.05
LP1	128.45	25.66	68.39	106.26	16.55
LP 2	116.38	24.58	30.69	123.95	17.41
LP 3	142.82	35.54	47.72	204.36	18.75
LP 4	108.37	24.92	36.45	180.55	19.51
LP 5	115.66	23.88	29.34	118.43	15.95
LP 6	120.45	28.44	34.55	162.86	14.33
LP 7	123.38	20.74	36.57	198.48	16.79
LP 8	118.48	20.36	38.35	174.16	17.63
LP 9	123.45	26.33	34.24	162.77	17.96
LP 10	106.34	29.34	29.39	89.65	16.72
Mean	121.33	26.34	38.72	154.23	17.15
LSD at 5% level	18.56	7.34	11.25	86.35	4.23

4.4. DISCUSSION

Potato (*Solanum tuberosum* L.) is one of the cheapest important vegetatively propagated crops of Bangladesh. Its production is increasing day by day because of its food value and other importance. The present work was undertaken to evaluate the potentials of the variability induction in potato plants through *in vitro* techniques and to justify its application in potato breeding programme. The field performance of four cultivars exposed to various tissue culture regimes was evaluated in the presence of appropriate controls. There were significant differences between cultivars and significant interactions between cultivars and treatments (Powell *et al.* 1989). Callus induction is a prerequisite on the way to generate somaclonal variation. To generate somaclonal variability, induction and establishment of fast growing embryonic calli are prerequisites. Because during callus division in artificial conditions different types of abnormalities occur in the genetic constituents who ultimately contributed to the regenerated plants (Larkin and Scowcroft, 1981; Shamima *et al.* 2003). As a result a lot of variation may be found in the plants regenerated from single callus population.

Callus is an actively dividing non-organized tissue of undifferentiated and differentiated cells often developing from injury (wounding) or in tissue culture (Pierik, 1987). Callus formed during *in vitro* culture has some similarities to tissue arising in vivo injury to plants (so called wound callus). However, there is difference in morphology, cellular structure, growth and metabolism between callus derived through tissue culture and natural wound cells. Now it has been well established that any tissue can be changed in to callus, if cultured on a suitably defined medium under controlled conditions. Skoog and Miller (1957) demonstrated hormonal control of differentiation and laid the foundation of clonal propagation of plants through tissue culture techniques. Since then, efforts have been made throughout the world to differentiate potato callus. At first Lam (1975) and Behnke (1975) were able to induce callus in potato. At CIP, callus culture was started in 1975. Wareh *et al.* (1989) were able to initiate callus, shoot regeneration and micropropagation of three potato cultivars. An exogenous supply of growth regulators is often recommended to initially callus induction from different explants. Exogenous supplies of auxin and often in combination with cytokinin to medium are essential for callus induction. Rao and Lee (1986) reported that

intermediate levels of auxin and cytokinin usually promote callusing in potato. However, many factors such as genotypes, compositions of the nutrient medium, physical growth factors such as light, temperature, humidity and endogenous supply of growth regulations are important for callus induction (Pierik, 1987).

In the present investigation MS medium was found to be effective for callus induction and plant regeneration, which is in agreement with the findings of Reddy and Reddy (1993).

Present study of callus induction was conducted with explants from *in vitro* meristem derived plantlets of four potato cultivars. For this purpose MS medium supplemented with different concentrations and combinations of auxin and cytokinin were used for callus induction and regeneration of shoots from callus. Two types of explants (internode and leaf segment) of four potato varieties were used from *in vitro* grown plants. In the present investigation, explants were cultured on MS medium supplemented with different concentrations of 2, 4-D, NAA and in combinations of NAA with BA in order to find out the most suitable culture media formulation for rapid induction of callus and plant regeneration from callus.

In Lal Shill, Patnai and Shill Bilati among all treatments of 2, 4-D highest callusing rate was found in medium having 3.0 mg/l from internodal explant. In Lal Pakri highest callusing rate was recorded in media having 2.5 mg/l 2, 4-D from internodal explants. In general, 2, 4-D formulations were found more effective in callus induction then those of NAA. Many workers observed 2, 4-D as the best auxin for callus induction as common as in monocot and even in dicot (Evans *et al.* 1981; Lu *et al.* 1982; Ho and Vasil, 1983; Jaiswal and Narayan, 1985; Chee, 1990; Khatun *et al.* 2003; and Alsadon *et al.* 2004).

According to the results of the present study different concentrations and combinations of auxin and cytokinin (BA + NAA) were also found to induce callus from internode and leaf explants of different potato varieties. This result was similar to Nasrin *et al.* (2003) in potato. Khatun *et al.* (2003) also found same result when using 1.0 mg/l BA + 1.0 mg/l NAA in potato callus induction. Similar results were also reported by Kathari and Chandra (1984) in African Merigold.

From the above experimental results, it was clearly focused that MS medium supplemented with 2.5 mg/l and 3.0 mg/l 2, 4-D were the best formulation for callus induction in potato. However, Mamun *et al.* (1996) reported that 2, 4-D proved less effective when used alone for other plant species. Sultana (2001) used 2, 4-D alone for callus induction from internode and leaf explants of potato. She also obtained same results like Mamun *et al.* (1996) in three potato cultivars. However, Malamug *et al.* (1991) found better results when using 2, 4-D as a callus inducing plant growth regulator in potato callus induction. Khatun *et al.* (2003) and Nasrin *et al.* (2003) used 2, 4-D alone for callus induction and found better results in potato.

Calli derived from the two sources of explants (internodal and leaf segments) were subcultured for shoot regeneration in MS medium supplemented with different concentrations and combinations of BA + NAA and KIN + NAA. After 20-25 days of inoculation, shoot regeneration started. The media with different concentrations and combinations of BA with NAA and KIN with NAA both were found effective for shoot regeneration of four potato cultivars from internodal and leaf explants derived callus. Two successive phases of culture are required for inducing multiple shoots as reported by many earlier researchers (Conover and Litz, 1978; Litz and Conover, 1981).

Between two combinations used BA + NAA was proved to be more effective than KIN + NAA for multiple shoot regeneration from leaf explants derived calli of potato varieties. Among the different combinations media fortified with 3.0 mg/l BA + 0.1 mg/l NAA were found optimum for maximum proliferation of shoot from leaf explants and media supplemented with 3.0 mg/l BA + 1.0 mg/l NAA were found most effective for shoot proliferation from internodal explants.

In Lal Shill the maximum leaf derived calli responded to develop shoots in medium having 3.0 mg/l BA + 0.1 mg/l NAA. The maximum number of shoots per callus was recorded in this medium formulation. Again in Lal Shill, the highest internodal derived callus responded to develop shoots in medium having 3.0 mg/l BA + 1.0 mg/l NAA. The maximum number of shoots per callus was recorded in the same medium formulation. Khatun *et al.* (2003) also found the same results in potato.

Sultana (2001) used BA + NAA for multiple shoots proliferation and obtained maximum shoots when cytokinin and auxin were used in MS medium in *Crysanthemum morifoliam*. Sultana (2001) was also found same results in potato. The effect of BA with NAA on multiple shoots proliferation has also been demonstrated by Malamug *et al.* (1991) in *Crysanthemum morifoliam*.

When MS medium supplemented with different concentrations and combinations of KIN and NAA, the internodal and leaf explants of potato showed better performance for shoot induction than BA with NAA. The highest calli induced multiple shoots in media having 4.0 mg/l KIN + 1.0 mg/l NAA from internode-derived calli of all four varieties of potato. The highest number of shoots/callus and highest shoot length were also recorded in this supplements. This result is similar to Nasrin *et al.* (2003); Sultana (2001) and Shamima *et al.* (2003).

Suh and Park (1986) recorded that KIN with NAA in MS medium stimulated proliferation and elongation of shoots in garlic. Ratio of cytokinin and auxin seems to play an important role in the morphogenic differentiation of cultured explants as suggested by Murashige and Skoog (1962); Steward *et al.* (1969); Thomas and Street (1970); Pareek and Chandra (1981); Beck and Coponetti (1983); Haider (1992); and Shamima *et al.* (2003). This differential response of *Solanum* explants may be due to the genotypic differences of the plant material used in the present experiment.

Somaclonal variation among plants regenerated through callus culture was reported by Chandra *et al.* (1985). They observed that plantlets regenerated through callus culture were not genetically stable. In the present study statistically significant variations regarding some morphological characters were also observed among the different calliclones of four potato cultivars, which support the findings of Chandra *et al.* (1985). Somaclonal variations was also observed and reported in many other crops by many earlier workers (Shepard *et al.* 1980 and Scowcroft, 1977).

Findings of the present study elucidate that plant regeneration could be possible from the calli developed from internodal and leaf segments of *in vitro* grown plantlets of four cultivars of potato viz. Lal Shill, Patnai, Shill Bilati and Lal Pakri. Somaclonal variation as

predicted by other workers was also observed on yield and yield related characters among the different somaclones of four potato varieties.

4.5. SUMMARY

The present study was undertaken with a view to optimize *in vitro* propagation technique considering various culture aspects for callus induction, shoot regeneration from callus and field evaluation of four potato cultivars (Lal Shill, Patnai, Shill Bilati and Lal Pakri).

In the present study, callus induction was conducted with the explants from *in vitro* grown meristem-derived plantlets. For this purpose, MS medium supplemented with different concentrations and combinations of auxins and cytokinins were used for callus induction and plant regeneration from callus. Two types of explants (internodal and leaf segments) were used from *in vitro* grown plants. It was observed that 2, 4-D was the best auxin when used singly for callus induction in potato. In Lal Shill, Patnai and Shill Bilati highest callusing rate were observed at 3.0 mg/l 2, 4-D from internodal explant and highest callusing rate was found at 2.5 mg/l 2, 4-D from leaf explant in Lal Shill and Shill Bilati.

The combination of BA+NAA and KIN+NAA showed better performance for shoot regeneration of all potato varieties for internodal and leaf explants derived callus. Between these two combinations BA+NAA proved to be more effective than KIN+NAA for maximum shoot induction from leaf explant derived calli. Among various treatments, the media fortified with 3.0 mg/l BA+0.1 mg/l NAA and 3.0 mg/l BA+1.0 mg/l NAA were found to be the best formulations where 40% multiple shoots formation from leaf explants in Lal Shill, 50% in Patnai and 50% in Shill Bilati and 40% in Lal Pakri, were recorded. However, the highest number of shoots 6.2 after 30 days of culture was recorded in this medium. The highest 50% calli derived from internode explants induced to develop multiple shoots regeneration in medium having 4.0 mg/l KIN+1.0 mg/l NAA in Lal Shill. This medium formulation was found to be best formulation for multiple shoot formation for internodal explants derived calla of Lal Shill. Plant regenerated through callus culture displayed pronounced somaclonal variations for plant height, number of leaves/plant, number of tubers/plant, tuber weight/plant and starch content. This variability can be useful to select improved potato lines with enhanced starch content.

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Appendix I

Types of virus present in different potato varieties before heat treatment and meristem culture

Varieties	Temperature		
	PLRV	PVY	PVX
Lal Shill	39	37	58
Patnai	41	42	62
Shill Bilati	58	45	27
Lal Pakri	62	50	21
Sada Gutti	54	52	20
Challisha	50	41	22

Appendix III

Effect of heat shock temperatures on the establishment of virus free meristem culture of six IPVs.

Varieties	Temperature		
	37 °C	42 °C	47 °C
Lal Shill	15.5	35.7	60.3
Patnai	17.4	40.3	74.5
Shill Bilati	0	9.2	15.4
Lal Pakri	0	7.3	13.1
Sada Gutti	0	0	17.2
Challisha	0	0	14.6

Appendix IV

Percentage of successful *in vitro* produced plantlets of different potato varieties in the field

Varieties	Percentage of successful plantlets
Lal Shill	80
Patnai	85
Shill Bilati	72
Lal Pakri	56
Sada Gutti	67
Challisha	77