STUDIES ON PHYTOHORMONE REQUIREMENTS FOR REGENERATION FROM POTATO EXPLANTS

A

THESIS REPORT

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Biotechnology

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled, **"STUDIES ON PHYTOHORMONE REQUIREMENTS FOR REGENERATION FROM POTATO EXPLANTS"** in partial fulfillment of the requirement for the award of the degree of Master in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala, is an authentic record of my own work during the period of five months from January 2006 to May 2006, under the guidance of **Dr. N. Das**, Head of the Department, Thapar Institute of Engineering and Technology, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled, **"STUDIES ON PHYTOHORMONE REQUIREMENTS FOR REGENERATION FROM POTATO EXPLANTS"** submitted by Karuna Kumra in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar Institute of Engineering and Technology (Deemed University), Patiala is a record of student's own work carried out by her under my supervision and guidance. The thesis has not been submitted for the award of any other degree or certificate in this or any other University or institute.

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ABSTRACT

In the present thesis work, attempts have been made to study and adopt different regeneration protocols for indigenous potato cultivars. Different phytohormones employed for the purpose include auxins like IAA (Indole acetic acid) and NAA (α naphthalene acetic acid), cytokinins like BAP (Benzyl amino purine) and zeatin, and gibberellic acid (GA_3). The potato cultivars chosen for regeneration studies were Kufri chipsona- 1 (CS-1), Kufri chipsona-2 (CS-2), Kufri jyoti (KJ), Kufri chandramukhi (KCM). These varieties are well adapted to the agroclimatic conditions of our country. The internodal stem segments of potato cultivars were chosen as explants because of their lesser sensitivity towards injuries while manipulation during regeneration studies. Initially, the media employed for regeneration consists of phytohormones like NAA, BAP and GA_3. But the response amongst the cultivars was not promising. The possible chances of occurrence of somaclonal variation because of the longer period of incubation of intervening callus phase made the indirect mode of regeneration an undesirable process. The promising results of using zeatin as a cytokinin in the regeneration media as cited in previous studies prompted us to incorporate zeatin in regeneration media for our indigenous potato cultivars. Media composition with different concentration of zeatin in combination with IAA and GA_3 were designed in this study. The introduction of zeatin in regeneration media resulted in minimum callus phase and accelerated shoot bud formation. Although the presence of zeatin in the media resulted in development of highly organogenic microcalli but the response was not same in all the cultivars. Out of all the cultivars studied, Kufri Chipsona (CS-1) showed best results. So, attempts need to be made further to modify the media for relatively rapid and efficient regeneration.

CONTENTS

1. INTRODUCTION

Potato *(Solanum tuberosum* L.) is one of the prominent crops capable of nourishing the world's population. Potato is exceeded only by wheat, rice, and maize in world production for human consumption (Ross, 1986). Potato tubers give an exceptionally high yield per acre many times that of any grain crop (Burton, 1969) and are used in a wide variety of table, processed, livestock feed, and industrial uses (Feustel, 1987; Talburt, 1987). The potential of the potato crop was realized in India soon after independence and the Central Potato Research Institute, Shimla was established in 1949 and took a leading role for the improvement of potato crop through conventional breeding techniques.

Potato crop occupied an area of 1.2 million hectare in our country with a total production of 23.5 million tones. It is a perishable commodity and its harvest time (March-April) coincides with the rise in temperature in the plains. Now when the country's population has crossed one billion mark, there is an urgent need to redesign the potato crop for quality and quantity improvement.

1.1. TAXONOMY AND GENETICS

Potato belongs to the genus *Solanum* (family *Solanaceae*) that consists of about 2,000 species. Within this genus, the section *Tuberarium* (Correll, 1962), also known as section *Petota* (D'Arcy, 1972), includes the tuber-bearing members, of which the cultivated potato is best known. *Solanum tuberosum* is a hybrid between the diploid species *S. stentotomum* and the diploid weed *S. sparsipilum* with subsequent chromosome doubling.

The potato has a series of ploidy levels, based on a haploid number of 12, ranging from diploid (2n=24) to hexaploid (6n=72), and including triploids, tetraploids, and pentaploids (Dodds, 1962). The cultivated potatoes are autotetraploid (4n=48); many wild species are diploid, but may range up to hexaploid. The tetraploid cultivated potatoes are not diploidized, so that there are four interchangeable genes at each locus (Ross, 1986).

The potato "seed" of commerce is not true botanical seed, but rather consists of sections of potato tuber with one or more "eyes", i.e. lateral buds (Everett, 1981). The potatoes of commerce are therefore all reproduced vegetatively, as clones. This necessarily means

that once a cultivar is produced, it is genetically stable in perpetuity, barring mutation, clonal variation or some other unusual event (Shepard *et al.* 1980).

1.2 EXTERNAL MORPHOLOGY OF THE PLANT

The potato is an herbaceous plant, 0.5-1 meter high. The leaves are alternate and irregularly pinnately compound. Inflorescence consists of several flowers. Flowers are pentamerous, actinomorphic, perfect, and have sympetalous colored corollas. Fruits are berries, absent in many cultivars (Burbank, 1921), and bicarpellate. Tubers form underground from rhizomes, from which adventitious roots are developed to become a fibrous mass (Burton, 1969).

1.3 PLANT TISSUE CULTURE – AN OVERVIEW

Tissue culture is the process whereby small pieces of living tissue (explants) are isolated from a plant and grown aseptically for indefinite period on a nutrient medium. Also tissue culture helps in propagation of useful germplasm under laboratory conditions. For successful plant tissue culture it is best to start with an explant rich in undetermined cells i.e. those from cortex or meristem, because such cells are capable of rapid proliferation. The usual explants are buds, shoot tips; root tips nodal segments or germinating seeds.
Plant tissue culture techniques are of use to nurserymen and plant propagators to achieve one or more of the following objectives:

- Elimination of viruses from infected plants
- Rapid multiplication of clones
- Vegetative propagation of species difficult to propagate
- All the year round propagation of clones
- Rapid multiplication of seedlings

An overview on various pathways leading to regeneration of plants through tissue culture is discussed here. Plant tissues regenerate *in vitro* through two pathways:
Organogenesis – is a developmental pathway in which roots or shoots are being induced to differentiate from a cell or cell clusters.
Embryogenesis: where usually single cell or a small cluster of cells undergoes differentiation to produce somatic embryos similar to zygotic embryos.

Morphogenesis could occur directly from the explants or indirectly via the formation of dedifferentiated callus. There are different pathways of regeneration such as:

- Indirect organogenesis from explants via callus
- Direct organogenesis from explants
- Direct embryogenesis from explants
- Indirect embryogenesis from explants via callus

The regeneration of complete plant via tissue culture has made it possible to introduce foreign genes into plant cells and recover transgenic plant.

For plant transformation, many different techniques such as *Agrobacterium*-mediated transformation, particle gun bombardment, microinjection, PEG treatment of protoplast and electroporation of protoplast can be used. However*, Agrobacterium*-mediated and particle gun bombardment are the most extensively used methods. Regeneration via the callus is amenable to *Agrobacterium*–mediated transformation while direct regeneration is amenable for particle gun bombardment (Chandra and Pental, 2003).

1.4 PLANT GROWTH REGULATORS:

Plant growth regulators are the critical media components in determining the developmental pathway of plant cells. The most commonly used are plant growth hormones or their synthetic analogs. There are several classes of plant growth regulators- auxins, cytokinins, gibberellins, ethylene, and absciscic acid. They all play a distinct role in development of plant.

For plant cells to develop into a callus it is essential that the nutrient medium contain a balanced ratio of plant hormones, i.e. auxins, cytokinins and gibberellins. The phytohormone balance varies for different tissue explants from different parts of the same plant and for the same explant from different genera of plants. Thus, there is no ideal media. Most of media in common use consist of inorganic salts, trace metals, vitamins, organic nitrogen sources (glycine), inositol, and sucrose and growth regulators. Relatively high ratio of auxin to cytokinin is required for root induction and high ratio of cytokinin to auxin is required for shoot induction from callus.

There are various factors that affect regeneration in plants such as position of the explant on the plant as well as size of explant, genotype of the explant, physiological state of the donor plant and explants, concentration of nutrients and phytohormones /

plant growth regulators in the culture medium and environment under which cultures are grown i.e. light, temperature, and humidity (Phillips and Hubstenberger, 1998).

1.5 MICROPROPAGATION OF POTATO PLANT

The term propagation means the generation (reproduction) of plants by asexual means. Micropropagation is the term used for propagation in laboratory. It is carried out in controlled and aseptic conditions, rather than in greenhouse or nursery. Micropropagation allows the rapid propagation in limited space under defined growth conditions (temperature, light) and microbiologically sterile plants are produced.

In case of potato, micropropagation is usually carried out by the following methods:

Shoot Tip Culture: Most micropropagation methods use apical or lateral shoot tips containing meristems. Excised shoots can be cultured on culture media with high concentration of cytokinin, which allows large number of axillary shoots to develop. Each of these axillary shoots can be separated and inoculated on root inducing medium so that complete plantlet is developed.

Culture of Nodes: In certain plants, it is not easy to generate multiple shoots using cytokinin. So, nodes comprising axillary buds are excised from the plant and placed in culture medium and allow the axillary buds to grow into shoots. Each node of these shoots is again subcultured. This serial subculturing could lead to generation of 2000 plants from a single node in a period of three months. This method of micropropagation is used for routine subculturing of germplasm of potato cultivars in our laboratory.

Regeneration by Organogenesis and Somatic Embryogenesis: Tissues from leaves, stems, roots and inflorescence can be cultured to promote direct morphogenesis or indirect morphogenesis to obtain either direct shoot generation (organogenesis) or somatic embryos (somatic embryogenesis) (Balasubramanian *et al.* 1998). It has been observed from previous studies that plants regenerated via organogenesis are more amenable to transgenic techniques. Organogenesis could be direct or indirect with reference to the intervening callus phase. In **direct organogenesis**, the roots and shoots are directly induced from cell under specific ratio of plant growth regulators. Direct regeneration is more favourable with respect to transgenesis. The reduced callus phase decreases the chances of occurrence of undesired somaclonal variation in regenerated plantlets. In **indirect organogenesis**, the intervening callus phase is

present. The technique of callus (tissue) culture was first developed in the late 1920s and 1930s and was one of the primary methods of tissue culture for many years.

Indirect organogenesis is based on the ability of the highly mature and differentiated cells to regress to a meristematic state. The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated cell mass called as callus is termed as dedifferentiation. The cells first undergo certain changes to achieve meristematic state these include replacement of non-functional cellular components damaged by lysosomal activity during the processes of cytoquiescence. Under defined ratio of plant growth regulators, the meristematic cells redifferentiates to form plant organ or the whole plant and the phenomenon is known as redifferentiation and and is followed by the process of induction of roots and shoots in callus cultures that is referred as organogenesis**.** The inherent capacity of a plant cell to give rise to a whole plant, which is retained even after a cell has undergone final differentiation in the plant body, is known as cellular totipotency (Bhojwani and Razdan, 1992).

Alternatively, callus cultures can be induced to undergo an entirely different development process under certain nutritional and hormonal conditions, which is known as somatic embryogenesis. In this process the callus cells undergo a pattern of differentiation similar to that observed in zygotes after fertilization and produce embryoids. Such somatic embryoids differ from normal embryos in being produced from somatic cells instead of fusion of two germ cells. In case of direct somatic embryogenesis, embryos develop directly on the excise plant tissue without an intervening callus phase. In the case of indirect embryogenesis callus is first generated from the excised plant tissue from which suspension cultures are raised and somatic embryogenesis is induced in cells in these suspension cultures. Indirect somatic embryogenesis is commercially attractive because very rapid scaling up is possible and potentially vast numbers of somatic embryo’s can be generated in small volumes of culture media in a synchronous and reproducible manner (Kumar and Kumar, 1996).

1.6 IMPORTANCE OF TRANSGENIC TECHNIQUES IN THE IMPROVEMENT OF POTATO CROP

Plant biotechnology is oldest of all the biotechnologies, which aims at improving the crop productivity. Classical plant biotechnology is limited to the introduction of required

characteristics into plants by genetic cross during sexual reproduction. This classical approach is useful if the desired genetic improvement is encoded by single gene. The most serious limitation of classical plant breeding technology is the time needed to develop a usable plant variety; it takes even many years to introduce a new variety. But genetic engineering has provided the technology with which plants can be genetically modified by manipulating plants own genes and also by introducing genes from taxonomically unrelated plants and other organisms such as bacteria, fungi, viruses and even animals. Genetic engineering will pave the way for increased food production and allow modification of plants for industrial needs (Balasubramanian *et al.,* 1998).

In case of potato, many varieties have been improved by conventional breeding which are resistant to late blight, ability to give more yield under short days and short growing periods of the plains, tolerance to viruses, immunity to wart disease, a moderate level of suitability for processing and resistance to cyst nematode. Currently transgenic techniques are considered to be the most efficient for various crop improvement programmes. In potato, the primary focus is on disease control, alteration in starch metabolism, improving nutritional quality. The nutritive value of potato is increased by cloning and expressing a non-allergenic seed albumin gene *AmA1* from *Amaranthus hypochondriacus* .The *AmA1* protein is non-allergenic in nature and is rich in all essential amino acids. The *AmA1* coding sequence was successfully introduced and expressed in tuber specific and constitutive manner. The expressed protein was localized in the cytoplasm as well as in the vacuole of transgenic tubers (Chakraborty *et al.* 2000).

Apart from above, there is now a growing interest to overcome cold storage problem of potato. Since reducing sugars accumulate at considerably high level if tubers stored at low temperatures (2^0C– 5^0C) – a process known as cold-induced sweetening. This leads to an unacceptable taste and color in the chips and crisps made from these tuber slices hence compromising the processing quality of tubers. Antisense technologies are being adopted globally to inhibit this undesirable phenomenon. There are many potato cultivars available suitable to varying agro-climatic conditions prevailing in our country. A comprehensive approach needs to be adopted to develop cultivars showing reduced cold sweetening phenotype in the tubers. This will help to promote potato processing sectors, farmers and the consumers.

There are certain other novel compounds being produced in transgenic potato. Cyclodextrins, a novel carbohydrate are being produced by inserting a chimeric gene coding for cyclodextrin glycosyltransferase (CGT) from bacteria. The enzyme produced in tuber will convert starch into cyclodextrins. The other approach is the production of hepatitis B surface antigen in transgenic potato for oral immunization. Nonetheless, increased resistance to pests and diseases is also an added feature of transgenic potato. Few of them are Bt potato for Colorado beetle management, transgenic potato resistant to potato virus X.

1.7 REGENERATION OF TRANSGENIC PLANTS:

To successfully incorporate the transgenic techniques in the improvement of quality and quantity of potato, regeneration studies are required to be made. Various methods of plant regeneration are available to the plant biotechnologists. Some plant species may be amenable to regeneration by a variety of methods, but some may be regenerated by one method only. Also not all plant tissues are suited to every plant transformation method, and not all plant species can be regenerated by every method.

Tissue culture and plant regeneration are an integral part of most plant transformation strategies. So key to success in integrating plant tissue culture into plant transformation strategies is the realization that a quick and efficient regeneration system must be developed. Organogenesis has been identified as the most efficient way for in vitro regeneration from a variety of transformed explants. It is desirable to regenerate a plant via direct morphogenesis rather than indirect. Plants regenerated from callus may show morphological variability.

- Sometimes this variability is a result of the culture conditions, such as the carryover effect of the plant growth regulator treatments.
- In other cases, the variability may be related to spontaneous genetic mutation during the culture process due to other factors.

Intervening callus phase while regeneration may lead to the dilution of character present in transformed explant or even its complete disappearance. Frequent cell divisions in the callus phase may lead to improper segregation and distribution of chromosomes accounting to somaclonal variation. So the media formulation should be such that the callus phase could be reduced.

2. LITERATURE REVIEW

A lot many studies have been made earlier too in view of regeneration and transformation of various potato cultivars. In one of the studies, an improved and efficient method has been developed for *Agrobacterium* mediated transformation of three varieties of potato namely Desiree, Bintje, Kaptah Vandel using internodal explants. The importance of zeatin riboside in the culture media, in controlling the development of highly organogenic microcalli has been demonstrated. The use of zeatin riboside in the regeneration media has allowed considerable reduction of callus phase and accelerated transgenic bud formation (7-9 buds per explant). Number of shoots per explant varies with the cultivar, Desiree and Bintje being more responsive than Kaptah Vandel. By combining the best treatment this protocol yielded an average transformation rate of 90% of treated longitudinal cut internodal explants. Short duration, high efficiency, genotype independence, low frequency of somaclonal variation make this system well suited for wider commercial applications of transgenic potato plants. The problem that has come across this protocol is that it does not work with same efficacy in all the three cultivars, Desiree and Bintje being more responsive than Kaptah Vandel (Beaujean and Sangwan, September 1998).

In other attempt, experiments have been carried for regeneration and *Agrobacterium* mediated transformation of two indigenous potato varieties of Bangladesh namely Jam Alu and Lal Pakri. Different concentrations of BAP (benzyl amino purine) and Kn (kinetin) were used separately to see their effect on multiple shoot regeneration. BAP showed better response in terms of no. of shoots per explant, no. of nodes, shoot length. Three different explants; leaf, nodal segment and internodal segments of two potato varieties were used to see their transformation ability. For this purpose, two strains of *Agrobacterium* LBA4404 (pBI121) and EHA105 (pCAMBIA 1301) were used. LBA4404 (pBI121) showed better results than EHA105 (pCAMBIA 1301) in all the explants. In both the varieties, leaf explants showed best transformation ability followed by internodal and nodal segments. Also out of two cultivars used in the study, Lal Pakri showed better response than Jam Alu (Sarker and Mustafa, June 2002).

A study has been made to observe the effect of culture medium on shoot initiation from calluses of different origin in potato. Attempts were made to regenerate shoots from calluses of different origin (tuber, leaf, internodal) on different media in two cultivars, Cv. Maris Piper and Cv. Desiree of potato. Media containing zeatin was found to be more

effective than the one containing BAP for shoot regeneration. Also Cv. Maris Piper showed better response than Cv. Desiree (Anjum and Ali, 2004).

In an attempt to improve the nutritional value of potato Chakraborty *et al* introduced a non allergenic seed albumin gene from *Amaranthus Hypochondriacus* in potato tuber. A specific potato regeneration media named PR was designed for regeneration of transgenic tubers. The media consists of MS basal media with 2.5 % sucrose, 1.5 mg/l BAP, 0.1 mg/l NAA and 0.1 mg/l GA_3. Internodal stem segments of potato cultivars under study were transferred to PR media where callus formation was initiated followed by its subsequent transfer to selective shoot bud induction media (Chakraborty *et al*., 2000).

It may be concluded here that a common regeneration protocol cannot be developed for all the cultivars. The variation in the requirements of phytohormones and media composition amongst the cultivars emphasizes on the need to develop and standardize regeneration protocol in a cultivar specific manner. This variation can be ascertained to factors mentioned below:

- Genotype of the explant: genetic make up of the cultivar leads to variation in responses.
- Type of the explant: Different types of explants used also contribute to variation in responses. The leaf, internode, tuber give different responses; leaf being more sensitive to injuries while manipulations during regeneration studies.
- Age of the explant: Younger tissues show better response than the older ones towards the regeneration protcol.
- Agroclimatic conditions: The different requirements of temperature, humidity, and photoperiod lead to the different responses amongst the cultivars.

AIM OF THE PRESENT STUDY:

Germplasms of the following cultivars being maintained in our laboratory namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Jyoti, Kufri Pukhraj, and Kufri Chandramukhi were chosen for the present thesis work. Experiments were carried out that could help directly or indirectly to adopt transgenic techniques for further improvement of potato crop. The main focus is to study requirement of media composition and phytohormones for direct and indirect regeneration from internodal stem segments of micropropagated potato plantlets.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 PROCUREMENT OF MATERIALS

The germplasm of various potato cultivars such as Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandramukhi (KCM), Kufri Jyoti (KJ) and Kufri Pukhraj (PR) are routinely maintained in our laboratory on modified MS medium.

The required chemicals were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, Qualigens Fine Chemicals, Merck, CDH Pvt. Ltd., New Delhi, and HiMedia Laboratories Mumbai.

3.1.2 MEDIA USED

Luria Bertani (LB) medium:

Yeast extract	- 0.5% (w/v)
Tryptone	- 1.0% (w/v)
NaCl	- 1.0% (w/v)
Agar	- 1.5% (w/v)

YEM Medium:

Yeast extract	- 0.4 (g/l)
Mannitol	- 10.0 (g/l)
$MgSO_4.7H_2O$	- 0.2 (g/l)
K_2HPO_4	- 0.5 (g/l)
NaCl	- 0.1 (g/l)
Agar	- 1.5%

3.1.3 MS BASAL MEDIUM (Murashige and Skoog Medium)

Components:

Major salts	concentration (mg/l)
KNO_3	- 1900
NH_4NO_3	- 1650
$MgSO_4.7H_2O$	- 370
$CaCl_2.2H_2O$	- 440

Major salts	
$MnSO_4.H_2O$	- 22.3
$ZnSO_4.7H_2O$	- 8.6
H_3BO_3	- 6.2
KI	- 0.83
$Na_2MoO_4.2H_2O$	- 0.25
$CuSO_4.5H_2O$	- 0.025
$CoCl_2.6H_2O$	- 0.025
Na_2Fe-EDTA	- 30.0

Vitamins and organics	
Myoinositol	- 100
Nicotinic Acid	- 0.5
Pyridoxine HCl	- 0.5
Thiamine HCl	- 0.1
Glycine	- 2.0
Sucrose	- 30.0 g/l
pH	- 5.8

Preparation of stock solutions of phytohormones:

For **Cytokinins*:*** Dissolve cytokinin such as BAP (Benzylaminopurine), zeatin in 0.5 N HCl, heat gently and made to the volume by adding sterile water. Adjust the pH to about 5.0. Concentration of stock solution of BAP was 2.0 mg/ml and zeatin is 2.5 mg/ml.

For **Auxins***:* Dissolved auxin such as IAA (Indole acetic acid) and NAA (α-Naphthalene acetic acid) in 95% ethanol or 1 N KOH, stirred gently and made up the volume by adding distilled water. Adjusted the pH to 5.0 and stored at 4^{o}C. Concentration of stock solution of IAA was 2 mg/ml and concentration of stock solution of NAA was 2.5 mg/ml.

For **gibberellins***:* Dissolved gibberellins like GA_3 in 95% ethanol, stirred gently and made up to the volume by adding sterile water.

3.1.4 DIFFERENT MEDIA USED

Different media formulations used for regeneration in different potato cultivars:

Media code	Basal media	Sucrose (%)	NAA (mg/l)	BAP (mg/l)	GA_3 (mg/l)	IAA (mg/l)	Zeatin (mg/l)
AOG	MS	3	0.01	0.01	0.25	---	---
A-1	MS	3	2.5	2.0	---	---	---
A-2	MS	3	---	2.0	0.1	---	---
A-3	MS	2	1.0	2.0	---	---	---
A-4	MS	3	0.5	2.0	---	---	---
ZIG	MS	2	---	---	3.0	0.05	3.0
ZIG-01	MS	2	---	---	3.0	0.05	2.0

All salts and additives are from HiMedia Labs Limited, India and growth hormones are from sigma chemicals, USA.

3.2 METHODOLOGY

3.2.1 MICROPROPAGTION FROM NODAL SEGMENTS

For direct regeneration AOG medium was used. The medium was prepared by adding all the constituent major and minor salts, vitamins and other organic constituents (MS-basal + 3% sucrose + 0.01 mg/l NAA + 0.01 mg/l BAP + 0.25 mg/l GA_3), the pH of the medium was set at 5.8 and then agar was added to it at concentration of 0.8% and then melted and dispensed in the tissue culture bottles (~ 50 ml medium in each bottle). These bottles were then autoclaved at 121ºC for 20 minutes at 15 psi, followed by their storage at 25ºC. Usually, inoculation was carried out after 1-2 days in order to check contamination problem.

As required in this study routine micropropagation was carried out using nodal segments from the following cultivars: **Kufri Chipsona-1, Kufri Chandramukhi, Kufri Jyoti and Kufri Pukhraj.** The various steps were followed as given below:

- The laminar airflow chamber was thoroughly cleaned with alcohol.
- Under aseptic conditions, young and tender plantlets of the above said varieties were taken out on a sterile glass plate, with the help of sterile forceps. These plantlets were not surface sterilized as these were already being maintained under *in vitro* conditions.
- Using a sterile scalpel, the roots of these plantlets were excised.
- The leaves were removed and finally the shoot part was cut into small segments, each segment retaining at least one node.
- Maintaining the correct polarity of the cut fragments, these were individually inoculated in the solidified AOG medium (About 10-12 such explants were inoculated per bottle).
- The bottles were sealed and labeled carefully and were finally kept in the culture room under maintained conditions of temperature (25ºC) and light (16 hrs. light, 8 hrs. dark) and the growth of the inoculated explants was monitored regularly.

3.2.2 REGENERATION USING INTERNODAL EXPLANTS

Internodal stem segments from micropropagated plantlets of different potato cultivars Kufri Chipsona-1, Kufri Chandramukhi, Kufri Jyoti and Kufri Pukhraj were used for regeneration studies. For this purpose, different media were used having the following codes: A1, A2, A3, A4, ZIG, ZIG-01. The exact composition of each medium is given in the section 3.1.4.

The media were prepared by adding all the components in required amounts. The pH of the medium was set at 5.8 and then agar was added to it at a concentration of 0.8% (w/v), then melted, and dispensed into tissue culture bottles. After that these bottles were autoclaved at 121^0C for 20 minutes at 15 psi followed by their storage at 25°C. After 2-3 days, after checking the media for any type of contamination, the explants were inoculated according to the following steps:

- The laminar airflow chamber was thoroughly cleaned with alcohol.
- Under aseptic conditions, young and tender plantlets of the above said varieties were taken out on a sterile glass plate, with the help of sterile forceps. These plantlets were not surface sterilized as these were already being maintained under *in vitro* aseptic conditions.
- Using a sterile scalpel, the roots of these plantlets were excised.
- The leaves were removed and the internodal segments were cut out from shoots.
- Internodal stem segments were individually inoculated horizontally on the solidified medium in bottles.
- The bottles were sealed and labeled carefully and were finally kept in the culture room under maintained conditions of temperature (25ºC) and light (16 hrs. light, 8hrs. dark) and the growth of the inoculated explants was monitored regularly.

4. RESULTS AND DISCUSSIONS

The focus of the present thesis work was to carry out direct and indirect regeneration studies from internodal stem segments of potato cultivars. First we discuss briefly on the features of some potato cultivars as used along with the media and phytohormone requirements for their routine micropropagation using nodal segments. Subsequent sections mainly deal with the indirect and direct regeneration studies from internodal stem segments.

4.1 MICROPROPAGATION FROM NODAL SEGMENTS

Kufri Chipsona-1, Kufri Chipsona-2, Kufri Jyoti, Kufri Pukhraj, Kufri Chandramukhi varieties were chosen for the study as they are suitable to the agroclimatic conditions of our country. The salient features of these cultivar varieties are discussed here:

Kufri Chipsona-1 is resistant to late blight and excellent for chip making and best grown in Indo-Gangetic plains.

Kufri Chipsona-2 is a medium maturing variety, resistant to late blight, excellent for chip making and is grown in Indo-Gangetic plains.

Kufri Chandramukhi is good for processing. It is best adapted to the north Indian plains and the plateau region of peninsular India.

Kufri Jyoti is good for processing, resistant to late and early blights and immune to wart, and tolerant to viruses, and wide adaptability.

For routine micropropagation nodal segments from the potato cultivars under study were transferred in a specific media named AOG instead of MS basal medium. The composition of AOG medium includes different phytohormones in specific concentration. It includes MS-basal + 3% sucrose + 0.01 mg/l NAA + 0.01 mg/l BAP + 0.25 mg/l GA_3.

The gibberellic acid (GA_3) helps in elongation of the internodes of the stem of the plantlet. As the internodal stem segments of these plantlets are further to be used in regeneration studies, so the use of these phytohormones is significant. Nodal explants with atleast one node from each of the above varieties were inoculated in the AOG medium and observed for growth. Direct morphogenesis led to the production of healthy plantlets. Rooting was initiated after 3 days and shoot initiation was observed usually after one week in all the nodal explants irrespective of the potato cultivars. After three weeks of inoculation healthy plantlets were observed. Fig. 1 and 2 shows three weeks old micropropagated plantlets of potato cultivars CS-1and CS-2.

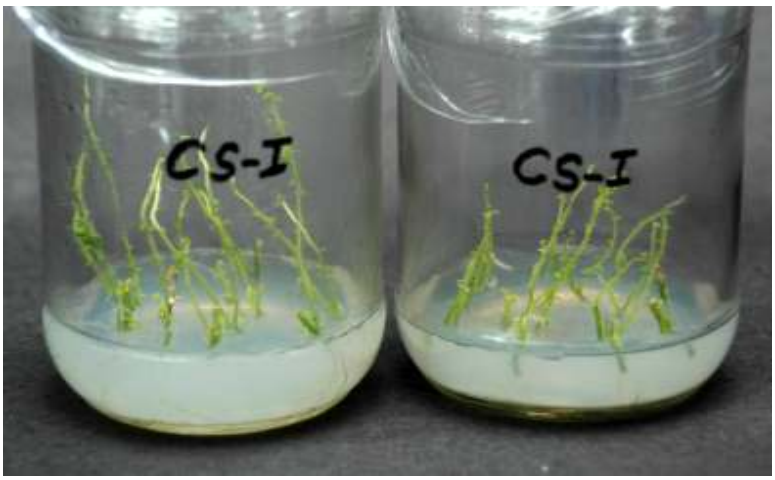

Fig. 1. Three weeks old micropropagated plantlets of potato cultivar **Kufri Chipsona–1**(CS-1) in AOG medium

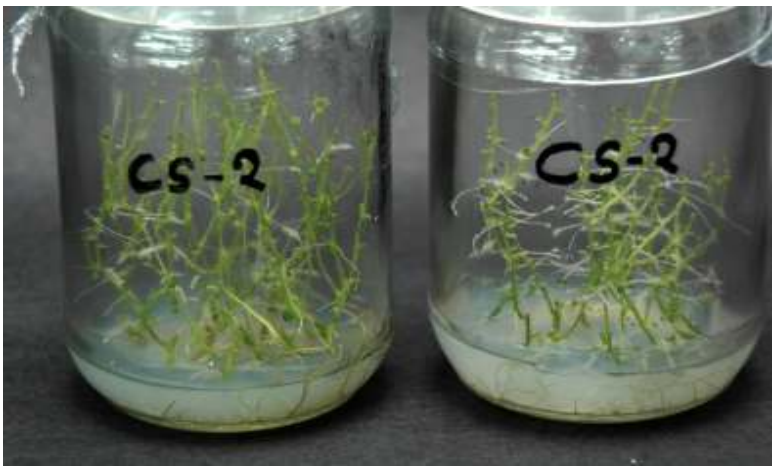

Fig. 2. Three weeks old micropropagated plantlets of potato cultivar **Kufri Chipsona-2** (CS-2) on AOG medium

4.2 REGENERATION STUDIES FROM INTERNODAL POTATO EXPLANTS

Attempts were made for regeneration using internodal stem segments from different potato cultivars namely: **Kufri Chipsona-1, Kufri Chipsona-2, Kufri Jyoti**, and **Kufri Chandramukhi**. The different phytohormones used in the preparation of media for regeneration are NAA, BAP and GA_3. Initially the following media were used for regeneration studies:

- **A-1 medium:** MS basal + 3% sucrose + 2.5 mg/l NAA + 2.0 mg/l BAP
- **A-2 medium:** MS basal + 3% sucrose + 2.0 mg/l BAP + 0.1 mg/l GA_3
- **A-3 medium:** MS basal + 3% sucrose + 1.0 mg/l NAA + 2.0 mg/l BAP
- **A-4 medium:** MS basal + 3% sucrose + 0.5 mg/l NAA + 2.0 mg/l BAP

A-1, A-3, A-4 media vary with respect to NAA concentration whereas concentration of BAP remains same. In order to study the growth response of internodal stem segments of different potato cultivars, the above media were employed for first transfer process separately. The varying concentrations of NAA were used to check their effect on intervening callus phase. Initiation of callus was reported from internodal stem segments of CS-1, CS-2, KJ, and KCM in the above media after one week. Four weeks old callus was then transferred to A-2 media. The presence of BAP (2.0 mg/l) as a cytokinin in A-2 media makes it a shoot induction medium. As it can be noted from Fig.3 and Fig.4 where callus initially raised in A-1 and A-4 media respectively was subsequently shifted to A-2 medium where shoot induction was observed after ~20 days. In potato cultivar Kufri Chipsona-2 (CS-2), callus formation took place in A-1 medium, callus is then transferred to A-2 medium after four weeks where it grows in size but no regeneration was observed even after 40 days as shown in Fig.5. In case of KCM callus growth ceased, and it turned to light brown color.

It is now clear that use of NAA in combination with BAP as used in A-1, A-3 and A-4 is not suitable for regeneration studies. Moreover, the different potato cultivars showed varying response in terms of callus initiation and subsequent shoot induction. The major drawbacks include (i) intervening callus phase, (ii) initiation of very less number of shoots from each callus (iii) and prolonged period of incubation with respect to shoot induction from callus. The prolonged process is often associated with undesirable somaclonal variations.

Fig. 3. Regeneration of potato cultivar Kufri Chipsona-1 (CS-1) from intervening callus phase using A-1 medium followed by A-2 medium. Internodal stem segments of potato cultivar CS-1 were placed in A-1 medium, callus initiation was observed after ~7 days, four weeks old callus was shifted to A-2 medium where callus grows in size, followed by second shifting to A-2 medium after 30 days where shoot regeneration was observed after ~ 20 days.

Fig. 4. Regeneration of potato cultivar Kufri Chipsona-1 (CS-1) from intervening callus phase using A-4 medium followed by A-2 medium. Callus initiated from internodal stem segments of CS-1 cultivar in A-4 medium was transferred to A-2 medium after four weeks where shoot regeneration was observed in ~25 days

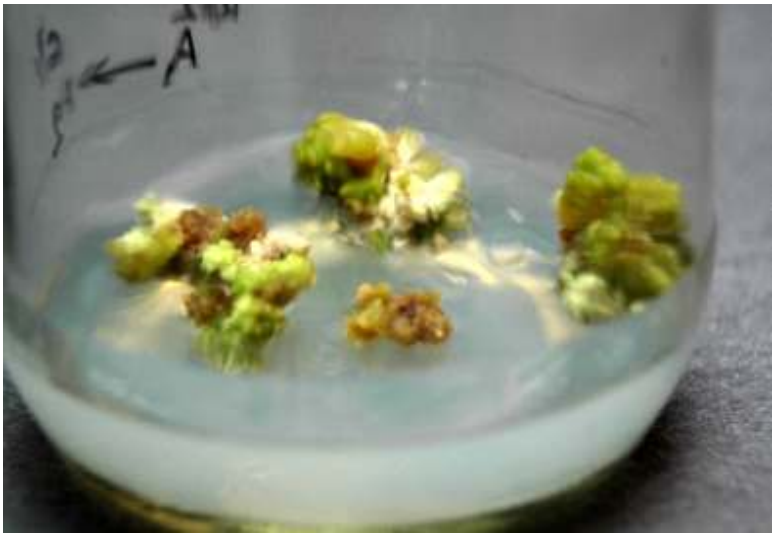

Fig. 5. Regeneration of potato cultivar Kufri Chipsona-2 (CS-2) from intervening callus phase using A-1 medium followed by A-2 medium. Four weeks old callus that initiated in A-1medium from internodal stem segments of CS-2 was shifted to A-2 medium where callus grew in size but no regeneration observed.

The poor response in the regeneration from internodal stem segments of the indigenous potato cultivars in A-1, A –2, A-3, A-4 media made us to look for some more efficient protocols. The promising results of using zeatin and zeatin riboside as a cytokinin in regeneration media of several other potato cultivars as cited in the literature prompted us to incorporate zeatin in regeneration media. **Beaujean** and **Sangwan** demonstrated in their study that use of zeatin riboside in the media allowed considerable reduction in callus phase and accelerated shoot bud formation from the callus. They employed zeatin riboside in combination with 2,4-D and GA_3. In the present study, we used only zeatin as a cytokinin. The concentration of zeatin riboside used by them is 0.8 mg/l whereas we used 3.0 mg/l and 2.0 mg/l of zeatin in the present study. The composition of media named ZIG and ZIG-01 with different concentration of zeatin in combination with other phytohormones like IAA as auxin and GA_3 as gibberellin are mentioned below:

- **ZIG medium:** MS basal + 2% sucrose + 3.0 mg/l GA_3 + 0.05 mg/l IAA + 3.0 mg/l zeatin
- **ZIG-01 medium:** MS basal + 2% sucrose + 3.0 mg/l GA_3 + 0.05 mg/l IAA + 2.0 mg/l zeatin.

Internodal stem segments from all the four cultivars CS-1, CS-2, KJ, KCM were initially transferred to the above zeatin containing media separately. Callus formation was initiated from the internodal stem segments of all the potato cultivars chosen for study. In CS-1 cultivar, callus phase was much reduced as shoot initiation started after 25 days of transfer to ZIG and ZIG-01 media. Fig.6 shows the reduced and green callus just before the induction of shoot formation. Some internodal explants of CS-1 showed distinct shoot formation after three weeks of transfer to ZIG medium as shown in Fig.7. Increase in number of shoots can be observed in Fig.8 where oragnogenic microcalli initiated in ZIG-01medium were transferred to fresh ZIG-01 medium after five weeks of transfer. It is of interest to note multiple shoot formation in some of the CS-1 explants.

Explants from other cultivars CS-2, KJ, KCM inoculated in ZIG and ZIG-01 media did not show desirable response. Callus phase was although reduced in potato cultivar Kufri Jyoti (KJ) but shoot regeneration was reported in few segments only. Five weeks old callus formed from internodal stem segments of KJ in ZIG medium was transferred to fresh ZIG medium where multiple shoot formation was initiated as shown in Fig.9. As can be noted from Fig.10, the callus formed from internodal stem segments of potato cultivar Kufri Jyoti (KJ) is small in size but do not show regeneration. The time taken by shoots in KJ to regenerate is markedly more than it takes in CS-1 variety. In potato cultivar Kufri Chipsona –2 (CS-2), callus initiated in ZIG medium but turned to light green color after a period of three weeks as shown in Fig.11.

The above data helps us to conclude that the use of zeatin in the media has allowed the duration of callus phase to be reduced and more importantly, formation of large number of shoot buds in a shorter time and without the somaclonal variation to be induced. On the contrary, the media containing zeatin that are ZIG and ZIG-01 did not show common response in all the potato cultivars under study. The experiments of *in vitro* regeneration showed considerable reduction in callus phase in all the potato cutivars chosen for the study but shoot bud induction was observed in few only. Out of all the cultivars used CS-1 showed best results in terms of early regeneration.

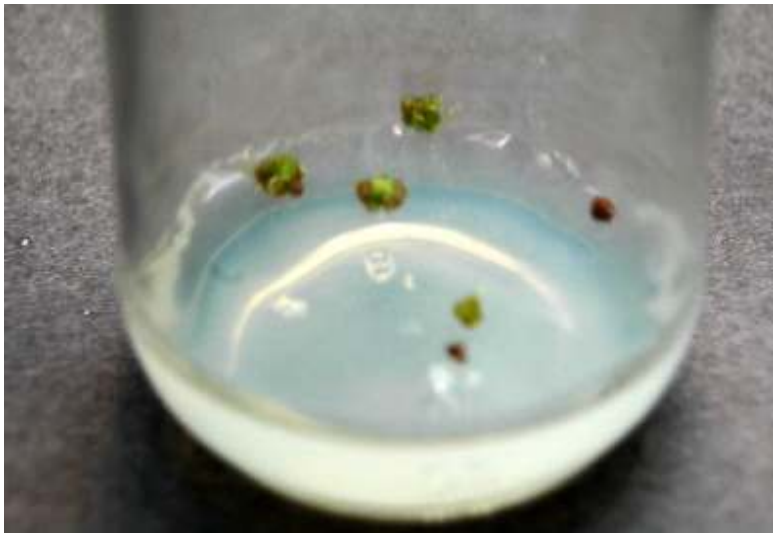

Fig. 6. Regeneration of potato cultivar Kufri Chipsona-1 (CS-1) with minimum intervening callus phase using ZIG-01 medium. Reduced and green callus just before the onset of shoot regeneration can be seen in this figure.

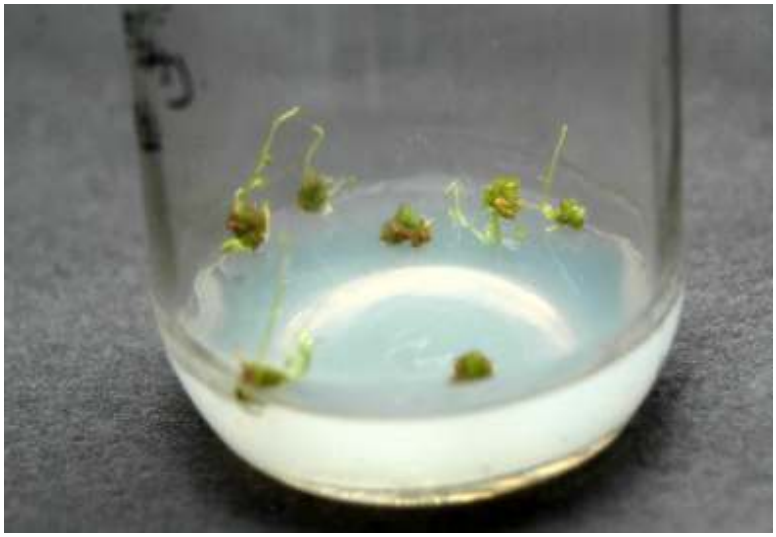

Fig. 7. Regeneration of potato cultivar Kufri Chipsona-1 (CS-1) with minimum intervening callus phase using ZIG medium. Green callus with multiple shoot regeneration is seen here after ~20 days of transfer.

Fig. 8. Regeneration of potato cultivar Kufri Chipsona-1 (CS-1) with minimum intervening callus phase using ZIG-01 medium. Internodal stem segments were placed in **ZIG-01** medium, shoot formation initiated from callus ~25 days, followed by transfer to fresh **ZIG-01** medium after 40 days where increase in shoot size was observed.

Fig. 9. Regeneration of potato cultivar Kufri Jyoti (KJ) with minimum intervening callus phase using ZIG medium. Five weeks old callus formed from internodal stem segments of KJ variety in ZIG medium was transferred to fresh ZIG medium where multiple shoot formation was initiated in a incubation period of 25 days.

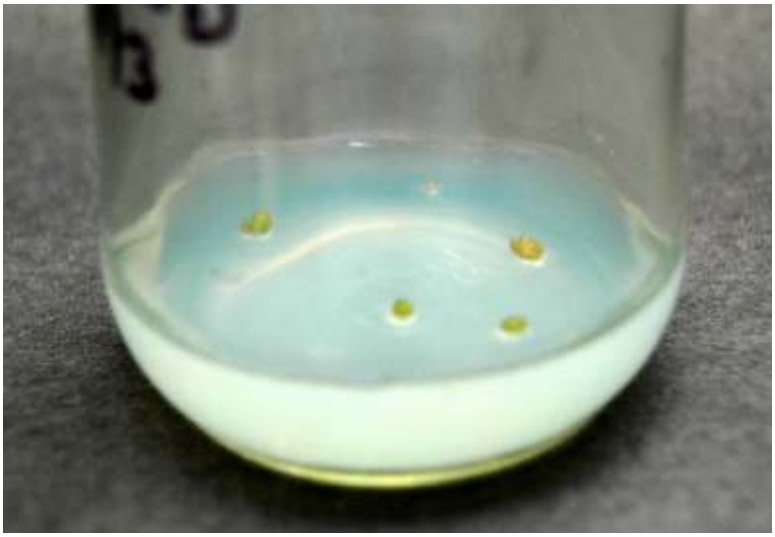

Fig. 10. Regeneration of potato cultivar Kufri Jyoti (KJ) with minimum intervening callus phase using ZIG medium. Callus formed from internodal stem segments of KJ variety was placed in ZIG medium turns to light green in 25 days and no regeneration was observed

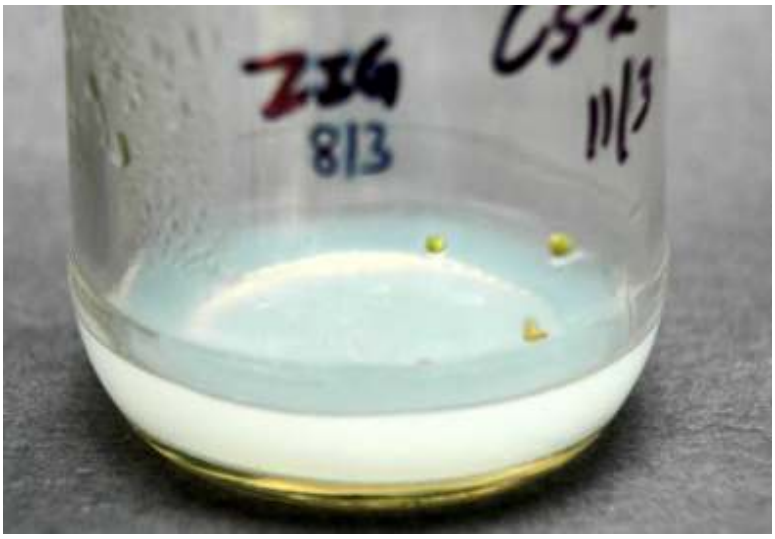

Fig. 11. Regeneration of potato cultivar Kufri Chipsona-2 (CS-2) with minimum intervening callus phase using ZIG medium. Callus formed from internodal stem segments in ZIG media, turned to light green color in ~20 days and no regeneration was observed

The regeneration studies suggests that explants showing multiple shoots are required to be transferred to zeatin containing medium further to ensure substantial growth. Thereafter, they could be easily transferred to AOG medium for normal vegetative growth. In some of the cases, if we transfer explants immediately after shoot initiation to the AOG medium, it affected the further growth (data not shown). So, the regeneration in potato cultivars Kufri Chipsona-1 (CS-1) and few of the Kufri Jyoti (KJ) internodal stem segments was checked further by shifting to AOG medium used for routine subculturing. The regenerated shoots were shifted to AOG medium where they develop into healthy plantlet after a period of 25 days as can be seen in Fig.12 and 13.

Now that it is apparent that the same regeneration protocol does not work with same efficacy in all the cultivars, we need to develop protocols for early and direct regeneration in different potato cultivars. Also it can be concluded that the requirement of phytohormones and media composition is different for different potato cultivars. So, the attempts need to be made further to modify the media for relatively rapid and efficient regeneration in other cultivars too. The regeneration studies as carried in this thesis work would help in genetic improvement of the potato crops through transgenic means.

Fig. 12. Micropropagated plantlets of potato cultivar Kufri Jyoti (KJ) in AOG medium from regenerated shoots in ZIG medium. The shoots regenerated in ZIG medium were separated and shifted to AOG medium, which grows into healthy plantlets after four weeks.

Fig. 13. Micropropagated plantlets of potato cultivar Kufri Chipsona-1 (CS-1) in AOG medium from regenerated shoots in ZIG medium. The shoots regenerated in ZIG-01medium were separated and shifted to AOG medium, which grows into healthy plantlets after four weeks.

5. SUMMARY

Potato is one of the agronomically important crops in the world. Currently transgenic techniques are used for quality and quantity improvement of the potato crop. Keeping in view the importance of regeneration studies in crop improvement the following basic experiments were designed and executed in the thesis work.

- Attempts were made for regeneration through organogenesis in various Indian potato cultivars. The cultivars chosen for study are: Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandermukhi (KCM), and Kufri Jyoti (KJ). Initially, the regeneration media named A-1, A-2, A-3, A-4 were developed based on phytohormones like IAA, NAA, BAP and GA_3. The internodal stem segments transferred to these media develop into callus in all the potato cultivars under study but shoot bud initiation was observed in few only. The intervening callus phase, less number of shoot buds for each callus and long incubation period made this regeneration protocol unfavorable one.

- Further, attempts were made to develop regeneration media with zeatin as a cytokinin in combination with IAA and GA_3. The media named ZIG and ZIG-01 were employed for transfer of internodal stem segments from all the potato cultivars chosen for study. The importance of zeatin was observed in the form of reduced callus phase and accelerated shoot bud induction. Reduced callus phase was observed in all the cultivars. Multiple shoot regeneration as observed in potato cultivar Kufri Chipsona-1 (CS-1) and Kufri Jyoti (KJ).

- The observations made help us to conclude that the potato cultivars vary with respect to requirements of media composition and phytohormones. Also it can be said that it is very difficult to generalize the protocol of regeneration because the process of organogenesis is influenced by various factors: age of the explant; younger tissue showing better response than the older one, position of the explant, genetic make up, agroclimatic conditions of the cultivar used, nutrient and phytohormone concentration of the media.

Finally, the attempts made for regeneration in potato cultivars may be useful for the introduction and regeneration of agronomically important traits without the problem of somaclonal variation in potato and, therefore, would be beneficial for a large scale application to potato transformation.

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