# STUDIES ON PHOSPHORYLATION STATUS OF STARCH IN POTATO TUBERS

***(Solanum tuberosum L.)***

**A**
**DISSERTATION**

By

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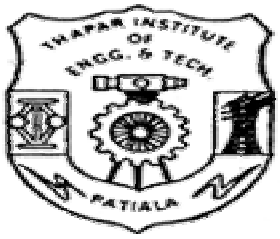
**Submitted in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology**

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## CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled,**" Studies on phosphorylation status of starch in potato tubers (*Solanum tuberosum L.)"***, in partial fulfillment of the requirement for the award of the degree of Masters 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 2003 to May 2003,under the supervision of Dr. N. Das,Assistant Professor, Thapar Institute of Engineering and Technology. I have not submitted the matter embodied in this dissertation, for the award of any other degree or diploma.

Place: Patiala

Date : ( ARADHANA MATHUR)

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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# DEDICATED TO .....MY GUIDE

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# 1. INTRODUCTION

The potato belongs to the family **Solanaceae**.The similarities in flower structure make the relation between these plants clear, although they are quite different in other characteristics. Today, in the world, one of the most important vegetables, are doubtlessly potatoes. The world production of potato is estimated to 307.8 million tonnes annually. Together with rice, wheat, and corn, potato integrates the four most important crops for mankind, not only by its huge production, but also it is a part of daily food consumption of almost all the inhabitants of the world.

## 1.1 ORIGIN

Archaeological evidence credits the natives of Peru with cultivating the earliest forms of potatoes approximately 4500 years ago.

The Spanish explorers of the 1500's were the first Europeans to come in contact with potatoes. They had ventured to South America, nearly 500 years ago, in search of gold, treasure, and new land. Along with the gold loot taken from South American natives, the Spanish conquistadors carried potatoes back to their homeland aboard their ships. By the19$^{th}$century, it spread throughout the continent, providing cheap and abundant food for the workers of the industrial revolution. Potato is now grown in probably all temperate countries and in tropical uplands.

The Solanaceae family is worldwide in distribution, but some members, such as tomato, tobacco, and potato, are native to South America and were unknown to Europeans before they began their explorations of the New World.

The name is derived from the Latin *solamen* which means "comforting," reflecting the sedative effects of some of the alkaloids produced by some members of the family. Deadly members of this family slowed the acceptance

of the edible members as food plants, but eventually they were discovered to be safe. Related alkaloids are present in the leaves of potato plants, causing digestive upsets, if eaten. If potato tubers are left in the light, they too will develop green color and alkaloids and such potatoes should not be eaten.

The familiar starchy "potato" is actually a **tuber** as reflected in the plant's Latin name, *Solanum tuberosum.*

**1.2 TAXONOMY**

Common potatoes (*Solanum tuberosum* L.) consists of 2 subspecies.

The most common cultivated species is a tetraploid, with a sporophyte chromosome number of 48(4n=48), which is thought to have arisen as a hybrid between the diploid species ***S. stentotomum*** and the diploid weed, **S. *sparsipilum*** with subsequent chromosomal doubling***.***

**1.3 GENERAL FEATURES**

At about the same time as potato plants begin to flower, small swellings start to develop at the ends of underground stems called **stolons**. These swellings are the new tubers, which store the excess food the plant produces during photosynthesis. It is a cool season, annual crop with optimum temperature ranging from 16ºC -20ºC for the best growth of tubers. They perform well on a variety of soils like sandy loams, silt loams, loams and peats and prefer a slightly acidic soil with a pH range of 5.8 to 6.5 .The soil should be evenly moist, but not wet or soggy for a good tuber growth***.***

The starchy tubers may be harvested and eaten at any time. The small, freshly harvested "new potatoes" are tender and delicious, but, to maximize yield, most potato growers wait until later in the season to harvest the potatoes. As the mature vines die above ground, the tubers develop an outer cork layer for survival in the soil during the winter. This layer also protects them from

desiccation (drying out) and wounding when they are harvested and put into **storage**.

Stored potatoes must be kept cool to prevent rotting by bacteria and fungi present on the tubers. They also need to be kept in the dark to prevent "greening" of tuber tissues.

Tubers grow underground and closer examination reveals that the eyes present on the tubers, are really buds in the axils of tiny scalelike leaves. Roots do not produce buds and leaves, so tubers are underground stems adapted to storage of nutrients. After several months in storage, the buds begin to sprout. Apical dominance can be observed on tubers as well as on aboveground stems. Because the buds at the apex of a tuber produce hormones that inhibit growth of lower buds, the sprouts are most developed at the apex of the tuber.

Most commercial potato growers and home gardeners do not plant the tiny seeds from the green berries produced after flowering. Potatoes are grown by **vegetative propagation**; that is, small tubers or pieces of tubers are planted. To maximize their planting stock, farmers may cut the tubers into several pieces. Each piece can grow into a new plant as long as an "eye" is present. Tuber pieces that do not have an eye are called "blind" and will not grow into a new plant. The cutting of tubers also releases the lateral buds from apical dominance, so that each eye may produce a plant. Vegetative propagation has several advantages. The tuber pieces, known as "seed" to farmers, contain substantial food reserves, so that a vigorous, green shoot pushes up through the soil more quickly than does the shoot from a tiny seed. In addition, each tuber piece grows into a plant that is genetically identical to the parent plant in color, taste, maturity, and other important characteristics. True botanical seed, from the fruit of a plant, is the product of sexual reproduction, which results in genetic variation. Each seed produces a plant that is slightly different from all of its siblings. **Genetic uniformity** is a great advantage when uniformity has an economic benefit, as in flowers, fruits, and ornamental plants, so vegetative propagation has become a common practice in modern agriculture. There are,

however, some disadvantages to vegetative reproduction. The advantages of uniform characteristics may be outweighed by the disadvantage of uniform susceptibility to pests and pathogens. If one plant in a field can be destroyed by disease, then so can all of its neighbours. Genetic uniformity increases the risk of loss. A second important risk lies in the large pieces of plant tissue that are planted during vegetative reproduction. Rather than a tiny seed, cuttings, roots, tubers, bulbs, and other relatively large plant pieces may be planted. These large plant pieces often carry pathogens, especially viruses and other systemic parasites, into the planting area at the very beginning of the season. The yield and quality of the crop may be greatly reduced due to their presence.

Only small collections of potatoes were brought from the New World in the early days of European potato production, and, from these tubers, further selections were made in an attempt to find potato **cultivars** suitable for consumption. Consequently, the genetic variation among the potato cultivars was very limited. Crop losses occurred every year from various causes, and occasional food shortages were not unusual. When crops were good, tubers were plentiful in the months after the harvest, but as the winter months passed, supplies diminished and the tubers began sprouting, ready to plant for the next crop.

## 1.4 MICROPROPAGATION AND CULTIVATION

The *in vitro* micropropagation methods are meant for rapid distribution and maintenance of existing cultivars and to bulk up new breeding lines for germplasm storage and transport. Furthermore, the molecular analysis of plantlets, which are propagated and stored *in vitro*, shows that micropropagation gives genetically stable plantlets . Another approach for micropropagation of potatoes is the *in vitro* induction of microtubers. The latter is an *in vitro* tuber of variable size and weight but which is much smaller than the minituber. Although considerable research has been performed on microtubers, their use on a commercial basis in the potato industry has been rarely formulated. However, the use of microtubers offers several advantages.

They can be stored several months and easily transported unlike *in vitro* plantlets. Thus, they are ideal for germplasm distribution as they can conform to long quarantine procedures. They can be directly sown into the soil. Unlike propagation of *in vitro* plantlets, they do not need subculturing to fresh media. They can be produced in bulk at any season.

Nevertheless, the cost of producing one microtuber is relatively higher than that of an *in vitro* plantlet and has thus restrained its use only for gene resource preservation Consequently, several experiments have been aimed on the factors controlling the tuberization process in view of implementing those findings in the development of rapid and cost effective methods of producing microtubers on a large-scale basis. These factors are environmental, hormonal, nutritional and physiological in nature.

The plants growing in vitro have tender, young roots lacking root hairs, which need to be hardened. Hardening and acclimatization comprises of transition from completely controlled conditions to uncontrolled field conditions.

## 1.5 NUTRIENT PROFILE OF POTATOES

Potatoes are particularly useful as source of energy and protein and are among the richest foods in potassium but poorest in Sodium .Its proteins are somewhat deficient in sulphur amino acids and Histidine. It is rich source of lysine.

| Constituents | Percentage (on tuber wt. basis) |
|---|---|
| Moisture | 50 - 81 |
| Protein | 1.0 - 2.4 |
| Fat | 1.8 - 6.4 |
| Starch | 8 - 29 |
| Non-starch Carbohydrates | 0.5 - 7.5 |
| Reducing Sugar | 0.5 - 2.5 |
| Ash | 0.9 -1.4 |
| Vitamins | 42.4mg /100g fresh wt |

## 1.6 POTATO PROCESSING

Fresh potato tubers are consumed as food and in recent years, demand of processed potato products such as potato chips, curls, flakes, fries etc. in the country has increased at a fast pace. However, not all potato varieties available in the country are suitable for processing

As rising temperatures in summers results in sprouting, microbial spoilage and senescence-induced sweetening, weight loss, rottage etc. Therefore, potatoes need to be stored at refrigerated cold stores.

### 1.6.1 NEED AND PROBLEMS

Potato plants are grown in moderate climate, therefore, continuous production of tubers is not possible round the year. As a result, major part of the potato tubers need to be stored at lower temperatures for a certain period of time to prevent damage caused by sprouting, microbial spoilage, rottage and weight loss. However, storage of potato tubers at low temperatures causes starch breakdown and accumulation of reducing sugars process called as cold-induced sweetening. The reducing sugars such as glucose and fructose, react with the amino groups of the proteins, a reaction called as Maillard reaction, which causes fried products to develop a dark brown colour along with an unpleasant and objectionable taste for the consumers. Therefore, due to the growing demand of the processed potato products, important parameters for the selection of raw material are:

Dry matter of the tuber: 21 to 23 %

Reducing sugar content: below 150 mg /100 g fresh tuber weight.

### 1.6.2 STARCH BIOSYNTHESIS

**Starch** serves as an important storage for carbohydrate residues. The breakdown of starch ensures retaining of essential life processes in the plants during periods of carbon shortage, as for example, during the night period in leave or in the potato tubers.

Plant cells contain a broad range of different but structurally very similar sugars. In this context, the questions how they are converted into each other and how the selectivity of the single reactions is safeguarded are of no small interest.

If not already activated, sugars have, like all other starting compounds of biosynthetic pathways at first to be activated. This occurs either by phosphorylation or by binding of a sugar residue to a nucleotide. Starch is generated by coupling glucose-1-phosphate to adenosine diphosphate.

It is believed that starch biosynthesis in potato tubers is a developmentally regulated process in the amyloplasts of the tubers. This process involves three enzymes: ADP Glucose pyrophosphorylase, Starch synthase, Starch branching enzyme. The enzyme ADP Glucose pyrophosphorylase, in particular, is a tetramer, composed of 50 kDa subunits. (Sowokinos J. R., 1976) The enzyme is subjected to strong allosteric control, strongly inhibited by inorganic phosphate whereas stimulated by 3-Phosphoglyceric acid (Sowokinos, 1981).

### 1.7 POTATO STARCH

Starch is next to cellulose, the most abundant carbohydrate molecule in the biosphere. It constitutes the main form for storage of energy in plants. It is produced by the polymerization of glucose residues, which again are products of photosynthesis. Since the plant is able to transport sugars from leaf to root or from leaf to seed and fruit, starch production can also take place in these organs. Different species produce starch grains of different shape. Since the shape of starch grains informs about their origin, they are helpful in the identification of seeds and other starch-containing plant parts.

The starch granule is composed of two polymers amylose and amylopectin. The amylose molecules are essentially linear á(1-4) glucan chains, whereas amylopectin molecules are highly branched and often contain small amounts of covalent bound phosphate.

*Alpha* - 1• 4 linkages are most common in starch though 1 •6 linkages do occur. Depending on the size of the molecule and its amount of 1 •6 linkages, it is distinguished between **amylose** and **amylopectin**. Amylose molecules are largely unbranched, water-soluble and contain mainly 1 • 4 linkages. They are, like most polysaccharides, meaning that the length of the molecule is not exactly defined, the number of glucosyl residues being between 200 and more than 1000. Depending upon the plant sources, the two forms are at different ratios. Amylose contributes the gelling property of starch and amylopectin contributes high viscosity.

### 1.7.1 MICROSCOPIC EXAMINATION OF POTATO STARCH

The granules of the potato starch vary greatly in size and shape: the largest are often egg-shaped and are visible to the unaided eye. The majorities are flattened ellipsoids and the smallest may be perfectly spherical. The granules may occur singly as well as compound granules consisting of two or three units. Granule size ranges from 15 to 100µm.

Due to the regular molecular structure of a starch grain (radial symmetry), certain sections of it absorb polarized light completely. Consequently, these zones look dark. The eccentric hilum towards the narrow end of the grain is normally well marked and surrounded by numerous concentric rings: these being very distinct on some grains.

### 1.7.2 PHOSPHORYLATION OF STARCH

Starch is composed of amylose and amylopectin. Amylose is a linear polymer whereas amylopectin being branched glucose polymer. In addition, amylopectin, depending on the plant organ where it is manufactured, contains different levels of phosphate monoesters. The phosphate groups are located as monoesters at the C-6 and at the C-3 positions of the glucose residues. The phosphate content in potato tubers is exceptionally high as compared with other plant storage organs. Starch biosynthesis is accomplished by different forms of starch synthase, which polymerize the glucose monomers using ADP-

glucose, and isoforms of branching enzyme, which introduce the branch points. Other enzymes are needed, to determine the final starch structure, e.g. the presence of a debranching enzyme is a prerequisite to synthesize the semi-crystalline starch granules.

# 2. REVIEW OF LITERATURE

In order to prevent spoilage, sprouting, weight loss, attack by pathogens, potato tubers need to be stored at low temperature which leads to accumulation of reducing sugars like glucose and fructose. This is known as cold-induced sweetening. Recent experimental evidence suggests that the phosphorylation status of the tuber starch has a correlation with this process of cold sweetening. Literature review mainly covers the above aspects.

## 2.1 POTATO (*Solanum tuberosum* L.)

Potato is an alien crop of the country. The importance of this crop was realized soon after independence. The Central Potato Research Institute, Shimla since its establishment in 1949,has been doing comprehensive potato breeding programme to develop improved cultivar varieties. They bred and released thirty-four high yielding varieties so far, which are suitable for different agro-climatic regions. Most of these varieties have been bred for consumption as fresh potatoes. However, potato varieties required for processing should have the following criteria: Tuber dry matter in the range of 21 to 23%, Reducing sugar content below 150 mg per 100 g fresh tuber weight and reasonably good yield to provide economic returns to the farmers (Singh *et al*., 1999).

In general, it was found that there is a higher variability in wild potatoes than in the cultivars, for various characteristics investigated as: dry matter content, starch content, protein content, starch yield and mean particle diameter (µm) of the starch granules (Jansen *et al.,* 2001)

## 2.2 STARCH IN POTATO TUBERS

Starch is the major predominant storage form in potatoes. Potato starch is, in many ways, a superior starch and its many interesting properties make it attractive for both food and industrial applications (Alexander,1995).

Starch is composed of two polymers amylose and amylopectin. The amylose molecules are essentially linear á(1•4) glucan chains, whereas the amylopectin molecules are highly branched and often contain small amount of covalently –bound phosphate (Hizukuri *et al*., 1970).

### 2.2.1 PHOSPHATE CONTENT IN STARCH

Potato tuber starch is characterized by a high content of phosphate relative to cereal starches. The phosphate groups are located as monoesters at the C-6 and at the C-3 positions of the glucose residues. In native potato starch, 0.3-0.4% of the glucose residues in the amylopectin are phosphorylated(Hizukuri *et al.*, 1970).

Due to the desired properties of the phosphorylated starches for industrial uses, autoclaving normal starches with phosphoric acid and urea chemically synthesize these. This chemical process is energy demanding and waste products are formed This low degree of phosphorylation has a profound effect on the qualitative properties of the starch changing e.g. the gelatinization temperature, viscosity, retrogradation and solubility. Due to the desired properties of the phosphorylated starches for industrial uses, autoclaving normal starches with phosphoric acid and urea chemically synthesize these. This chemical process is energy demanding and waste products are formed (http://www.plbio.kvl.dk/plbio/starch.htm).

### 2.2.2 BOUND PHOSPHATE

Majority of the phosphate is bound to the amylopectin fraction of starch (Hizukuri *et al.*, 1970). The starch –bound phosphate constitutes a major part of the total phosphate pool in the potato tubers (Quick *et al.*, 1976).

Small starch granules contain approximately 25% more bound phosphate per glucose residue than large granules whereas the overall level of phosphorylation does not depend upon the tuber size. (Nielsen *et al.,* 1994). The level of phosphorylation may vary approximately two folds among different potato cultivars and depends upon the growth conditions (Nikuni *et al.,* 1969).On an average, one of every 200-500 glucose residues is phosphorylated (Nielsen *et al.*,1994)

## 2.3 COLD-INDUCED SWEETENING - POSSIBLE EXPLANATIONS

Considerable progress has been made in studying the process of cold-induced sweetening in potato tubers. A few crucial enzymes are identified as well as the corresponding genes are also characterized a few genetically modified potato

plants have also been developed which are considerably promising. It is thought that the cold –induced hexose accumulation is caused by an imbalance between starch breakdown and glycolytic activity. Several explanations are also there related to cold-induced sweetening in the potato tubers.

(a) The starch is thought to be degraded mainly via the phosphorylytic route, based on the relative activities of phosphorylase and amylolytic enzymes. In other words, an increase in the activity of one or more starch degrading enzymes leads to the above process.

(b) An increased concentration of hexose phosphates as a result of cold-lability of phosphofructokinase and other glycolytic enzymes.

(c) Increased activity of the enzymes involved in sucrose synthesis.

(d) Increased activity of invertases.

(Isherwood 1973; Pollock and Rees, 1975; Guy *et al*., 1992; Deiting *et al*., 1998)

Interaction between ADPG pyrophosphorylase subunits contributes to the assembly and stability of the enzyme. Plastidial isoamylase plays an important role in determining starch structure; phosphoglucose trafficking from cytosol to amyloplasts and to chloroplasts affects the substrate availability for starch synthesis; and phosphorylation of starch influences starch degradation. (http://www.imb.sinica.edu.tw/imb/researcher/html)

## 2.4 STARCH PHOSPHORYLATION AND COLD- INDUCED SWEETENING IN POTATO TUBERS

Various attempts have been made currently in order to identify enzymes / proteins involved in starch synthesis and its modification. For this, starch-bound proteins were isolated from potato tuber and antisera raised against them for screening of potato cDNA expression libraries. Recently, a cDNA clone has been isolated and characterized. It is 4851 bp in size encoding starch-granule-bound protein subsequently called as R1 (1464 aa residues). To analyze the function of this protein, transgenic potato plants were

generated, showing reduced expression level of R1 gene using antisense RNA technology.

The R1 protein is involved in determining the phosphate content of potato starch. A major observation made is the reduction of the phosphate monoester content of the starch synthesized in the transgenic lines down to 10% as compared with wild-type plants. This indicates that the R1 protein is responsible for the phosphorylation of starch, which is also supported by the fact that the expression of the protein in *Escherichia coli* leads to elevated phosphate contents of the synthesized glycogen. Similarities to PEP synthase in the C-terminal part of the R1 sequence could be taken as an indication that R1 mediates an ATP–dependent starch phosphorylation by a Dikinase type of reaction

The biochemical mechanism by which the R1 protein phosphorylates amylopectin remains to be elucidated. It is not known which substrate acts as a phosphoryl donor, nor which type of glucans are phosphate acceptors. Evidence that phosphoenol pyruvate may act as a phosphoryl donor comes from slight sequence homology of the R1 protein to the PEP synthases from different bacteria.

The overall secondary strucuture of starch from transgenic tubers with lowered levels of R1 protein was altered, that led to less degradation during cold storage.It was found that the level of reducing sugars was upto nine fold less after two months of storage at 4ºC as compared to the wild type tubers. In other words, repression of cold sweetening was noted in transgenic tuber (Lorberth *et al.,* 1998).

In our country, no comprehensive biochemical and molecular approaches have been adopted so far to understand and inhibit the process of cold-induced sweetening in potato tubers. As we need to focus on the potato cultivars which are more suitable in our agro-climatic conditions. Few cultivar varieties in our country are given here namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri

Chandramukhi, Kufri Jyoti, Kufri Pukhraj, Kufri Ashoka, cultivar Desiree. Keeping in view with the above problem, the following **objectives** were formulated for the dissertation work:

1. Micropropagation, hardening and acclimatization of the above potato cultivars
2. Cultivation of the hardened plantlets in the trial plots for the production of mini tubers
3. Isolation of starch granules from various tubers
4. To determine total phosphorous content in various starch granule preparations and to make a comparison among different cultivars
5. To see the effect of temperature on the status of starch phosphorylation

# 3. MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 PROCUREMENT OF THE MATERIALS:

The germplasms of various potato cultivars such as Kufri Chandramukhi, KufriChipsona-1, Kufri Chipsona-2, Kufri Badshah, Kufri Ashoka, Kufri Pukhraj, Desiree were procured from Central Potato Research Institute (CPRI), Shimla.

Potassium dihydrogen phosphate ($KH_2PO_4$ ) was bought from Qualigens Fine Chemicals (ExcelaR).

Magnesium nitrate [(MgNO3) $_{2.}$6H2O] was obtained from CDH, Pvt. Ltd.

Ascorbic acid used was AR grade and obtained from CDH, Pvt. Ltd.

Ammonium Molybdate [ $(NH_4)_6.M_{O7}.O_{24}.4H_2O$ ] was obtained from Qualigens Fine Chemicals. [***Note:** All the chemicals used were of analytical grade (AR)].

### 3.1.2 MEDIA

**Shoot Multiplication Medium (SM-1):**

<u>MACRONURIENTS</u> (mg / L)

| | | |
|---|---|---|
| $KNO_3$ | = | 1900 |
| $NH_4NO_3$ | = | 1650 |
| $MgSO_4.7H_2O$ | = | 370 |
| $CaCl_2.2H_2O$ | = | 440 |
| $KH_2PO_4$ | = | 170 |

<u>MICRONUTRIENTS</u> (mg / L)

| | | |
|---|---|---|
| $MnSO_4.H_2O$ | = | 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 |
| $Na_2$Fe-EDTA | = | 30.0 |

ADDITIVES (per L)

| | | |
|---|---|---|
| Thiamine HCl | = | 0.4mg |
| Nicotinic Acid | = | 0.5mg |
| Pyridoxine HCl | = | 0.5mg |
| Ca-Pantothenate | = | 2.0mg |
| Glycine | = | 2.0mg |
| Myo-inositol | = | 100mg |
| Sucrose | = | 30 g |
| NAA | = | 0.01mg |
| BAP | = | 0.01mg |
| $GA_3$ | = | 0.25mg |
| Agar | = | 0.7% (w / v) |

The pH of the medium was set at 5.8.

### 3.1.3 PHYTOHORMONES

NAA (Naphthalene acetic acid) was prepared by dissolving in minimum amount of 1.0N KOH and finally making up the volume by distilled water.

BAP (Benzyl Amino purine) was prepared by dissolving in minimum amount of 1.0N HCl and finally making up the volume by distilled water.

Gibberellic acid was prepared by dissolving it in distilled water.

## 3.2 METHODS

### 3.2.1 PREPARATION OF SHOOT MULTIPLICATION MEDIUM:

1. SM-1 is a modified MS medium and was prepared by adding all the components in the required amounts as given in the section 3.1.2.
2. The pH of the medium was set at 5.8 and then agar was added to it at a concentration of 0.7 %(w / v).
3. This was then melted and ~50.0 ml of the media was dispensed in each tissue culture bottle.
4. These bottles were then autoclaved at 121º C for 20 minutes at 15 psi, followed by their storage at 25º C.

### 3.2.2 MICROPROPAGATION OF POTATO CULTIVARS:

1. Seven varieties– Kufri Chipsona-1, Kufri Chipsona-2, Kufri Ashoka, Kufri, Pukhraj, Kufri Chandramukhi, Kufri Jyoti, cultivar Desiree were micropropagated on SM-1 medium on a routine basis.
2. The laminar airflow chamber was thoroughly cleaned with alcohol and under aseptic conditions, young and tender plantlets of the above mentioned varieties were taken out on a sterile glass plate, with the help of sterile forceps.
3. These plantlets were not surface-sterilized as these were already being maintained under in vitro conditions.
4. Using a sterile scalpel, the roots of these plantlets were excised, the leaves were separated and finally the shoot was cut into small segments each segment retaining atleast one node.

5. Maintaining the correct polarity of the cut segments, these were individually inoculated in the modified SM-1 medium (About 6-7 such explants were inoculated per bottle).

6. These bottles were sealed and labeled and were finally kept in the culture room under maintained conditions of temperature (25º C) and light and the growth of the inoculated explants was monitored regularly.

### 3.2.3 HARDENING AND ACCLIMATIZATION

1. All the above rooted potato varieties maintained by tissue culture were gently washed with warm water to remove the agar from the roots.

2. These plantlets were treated with 1 % (w / v) Bavistin for 5 –10 minutes and were transferred to the plastic protrays containing the potting mix-Soilrite.

3. These plantlets were further shifted to highly humid conditions in a polyhouse for two weeks with the temperature maintained at 25º C and relative humidity of 85 to 90%.

4. These hardened plantlets were then shifted to shade house for 6-7 days with temperature maintained at 25º C and relative humidity of 65 to 70%.

### 3.2.4 CULTIVATION IN THE FIELD

1. These plantlets were then cultivated in the field in different bed for different varieties, with five plants in a row for each variety.

| | |
|---|---|
| Date of planting the crop | : 16.11.02 |
| Number of beds | : One |
| Length of each bed | : 18 m |
| Width of each bed | : 1m |
| Number of rows per variety | : five |
| Number of plants per variety | : ~50 |

2. Their growth was monitored regularly along with the application of water, fungicide and fertilizers whenever required.
3. After four months, minitubers from each variety were harvested, washed gently with water, dried on the folds of blotting paper and finally various studies were carried out on them.
4. The tubers used immediately after harvest were marked as freshly harvested (FH).
5. Other tubers were distributed to different temperatures of 4º C and 20º C, for three weeks, for subsequent use.

All the potato varieties were cultivated in the same agroclimatic conditions of sunlight, relative humidity, water balance, fertilizer dose etc.

### 3.2.5 STARCH GRANULE ISOLATION FROM POTATO TUBERS

The starch granules were isolated according to the following protocol:

1. Weighed 5.0 g of thin slices of peeled potato tubers and crushed them using pestle and mortar to form a paste; formed a uniform slurry using ice-cold distilled water.

2. Filtered the homogeneous slurry through a double-layered cheese cloth into a 150 ml conical flask and then transferred the contents into clean 50 ml centrifuge tubes and made up the volume to 50.0 ml using ice-cold water.

3. Incubated the tube in ice for atleast half an hour and then centrifuged the solution at 5000-6000 rpm for 10 minutes.

4. Decanted off the supernatant carefully and to the white coloured starch granules, added nearly 30.0 ml of ice-cold water, mixed thoroughly and centrifuged at 5000-6000 rpm for 10 minutes to give the first water wash.

5. Decanted the supernatant carefully and again added 30.0-ml of ice-cold water,

mixed and centrifuged at 5000-6000 rpm to give second water wash.

6. Similarly gave a third and fourth water wash and decanted off the supernatant carefully without damaging the pellet.

7. To the pellet added 20.0-ml of acetone, mixed and vortexed the solution and centrifuged at 6500 rpm to give the first acetone wash. Similarly gave two more acetone washes and finally decanted off the supernatant carefully.

8. Air-dried the starch granules by incubating the tubes at 37°C overnight.

9. Weighed the air-dried starch granules and quantified them per 100.0-g fresh tuber weight.

### 3.2.6 MICROSCOPIC STUDIES OF STARCH GRANULES

The granules of the potato starch vary greatly in size and shape, the largest are often egg-shaped and are visible to the unaided eye. The majorities are flattened ellipsoids and the smallest may be perfectly spherical.

1. Took a clean dry glass slide and put a drop of distilled water at the centre of the slide.

1. Put a minute drop of 0.1% $I_2$-KI solution on top of the water droplet and using a thin, dry brush, mounted the starch powder over the droplets.

2. Carefully, mounted the iodine-stained starch powder with a drop of glycerol and cover it with a glass cover slip without entrapping air bubbles in the field.

3. Study the stained starch powder at first 10 X and then 40 X, under the compound microscope.

### 3.2.7 EXPERIMENT TO CHECK THE PRESENCE OF ANY FREE PHOSPHATE IN STARCH SAMPLES

The phosphate content in potato tubers is exceptionally high as compared with other plant storage organs. The phosphate groups are bound as monoesters at the C-6 and at the C-3 positions of the glucose residues. Therefore, the phosphate present in the potato starch is the bound phosphate.

To check that whether there is any free phosphate in starch, experiment was carried out as follows:

1. Nearly 10.0 mg of starch powder was thoroughly suspended in water and kept for 20-25 minutes at room temperature, so that the free phosphate, if any, would come into the solution.

2. Then the suspension was centrifuged at full speed and the supernatant was assayed for phosphorus by Ascorbic acid–ammonium molybdate assay. However, the results showed that amount of free phosphate in the supernatant was negligible and that was almost equivalent to the blank sample.

### 3.2.8 DETERMINATION OF TOTAL STARCH-BOUND PHOSPHORUS

In this method, the phosphomolybdate complex is reduced by ascorbic acid. Essentially, this method is based on the principle of colorimetric estimation of inorganic phosphate. Inorganic phosphate reacts with ammonium molybdate in an acid solution to form phosphomolybdate complex, addition of a reducing agent like ascorbic acid, reduces the molybdenum, in the phosphomolybdate complex, to give a blue colour. In this reaction, the uncombined molybdic acid will remain unaffected. This ascorbic acid method is very sensitive, by which one can easily determine inorganic phosphate as low as 0.01 micromole.

Here, we need to determine the total starch-bound phosphate. For this ashing procedure of starch was adopted to determine bound phosphorus. Complete ashing enables the conversion of organic phosphates to inorganic phosphates. Basically, the ashing procedure was coupled with the very sensitive inorganic phosphate method.

**(A) REAGENTS**

**REAGENT A**: Ascorbic acid, 10%

Weighed 0.5 g of ascorbic acid and dissolved in 5.0 ml of distilled water.

**REAGENT B**: 0.42% Ammonium molybdate.$4H_2O$ in 1N $H_2SO_4$

Dissolved 0.042 g of ammonium molybdate in 0.286 ml of conc. $H_2SO_4$ and made up the volume to 10.0 ml.

**MIX**: Mixed 1.0 ml of reagent A and 6.0 ml of reagent B before use.

**STANDARD $KH_2PO_4$ (100 mg P in 1.0 liter solution)**

Weighed 0.439.5 g of $KH_2PO_4$ and dissolved in 400.0 ml of distilled water. Added 25.0 ml of 7.0N $H_2SO_4$ to it and made up the volume to 1.0 L.

**MAGNESIUM NITRATE:** Weighed 0.4 g of Magnesium nitrate [$(MgNO_3)_2.6H_2O$] dissolved in 4.0 ml of 95 % alcohol.

**OTHERS (as described in the methods):** 0.5 N HCl, Pipettes, Conical flask, Dispenser, Microfuge tubes, Micropipette, 0.1N $H_2SO_4$, Water bath, Microcentrifuge, Test tubes, Bunsen burner, Gloves, Weighing balance, Butter paper, spatula. Etc.

**(B) PROCEDURE**

1. 5.0 mg of each starch granule preparation from potato tubers was suspended in 50 µl to 100-µl water. The material was taken to dryness and completely charred over a strong flame of gas burner.

2. The tubes were then allowed to cool and 0.4 ml of 0.1N $H_2SO_4$ was added. The tubes were capped with marble and heated in boiling water bath for 10 minutes, to ensure solubilization as well as conversion of any pyrophosphate formed during ashing, to inorganic phosphate.

3. The solution was allowed to cool and then transferred to 1.5-ml microfuge tubes followed by centrifugation for further clarification. The clean supernatant was transferred to fresh microfuge tubes and 25.0 µl and 50.0 µl aliquots of the sample were taken for assay.

4. Sequential addition was done in the following way, for a reaction volume of 0.3 ml:

- 25.0 µl or 50.0 µl aliquots of the sample
- 150.0 µl of 0.1N $H_2SO_4$
- Made up the volume to 300.0 µl by addition of water
- In case of blank, 150.0 µl of 0.1N $H_2SO_4$ and 150.0 µl of water were mixed together.

5. To each reaction tube, added 0.7 ml of the MIX, vortexed the tube and incubated in water bath at 45°C for 20 minutes.

6. Cooled the tubes and read the absorbance at 820 nm.

* **NOTE**: For the preparation of the standard graph $KH_2PO_4$ solution was used and the following amounts of phosphorus

were taken as standards: 0.5 µg, 1.0 µg, 1.5 µg, 2.0 µg, 2.5 µg, 3.0 µg

## 4. RESULTS AND DISCUSSION

The present dissertation work deals with the micropropagation and cultivation of various potato cultivars, harvesting of the minitubers, isolation of starch granules from potato tubers of different cultivars, microscopic studies of the starch granules, determination of total starch-bound phosphorus from freshly harvested tubers as well as tubers stored at different temperatures and finally, status of starch phosphorylation in the tubers of different varieties were analyzed.

### 4.1 MICROPROPAGATION

The following potato varieties namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Ashoka, Kufri Pukhraj, Kufri Chandramukhi, Kufri Jyoti, cultivar Desiree were micropropagated on the shoot multiplication medium (SM-1), which is a modified MS medium. It contains various phytohormones such as auxin, cytokinin and gibberllin. The ratio of auxin to cytokinin is 1:1 with respect to absolute amount.

Explants with atleast one node from the above potato varieties were inoculated in the solid SM-1 medium. Profuse shoot growth was observed within two weeks of inoculation, along with the root growth to some extent. Young and tender leaves started appearing on the stem within the same time period. The growth of the inoculated explants was observed regularly and these were checked for contamination. No contamination in any variety was observed. Further subculturing was carried out depending upon the growth conditions of the inoculated explants and their growth was observed regularly.

### 4.2 HARDENING, ACCLIMATIZATION AND CULTIVATION

The micropropagated plantlets with tender roots were hardened and acclimatized in the potting mix-Soilrite for two weeks. Soilrite consisted of peat moss to absorb water and vermiculite along with perlite helps in

aeration. While this step was carried out, the plantlets were kept under high humid conditions, to prevent desiccation.

Finally, these were planted on the trial plots, within our own campus upon eight beds of equal size. Weeds were manually removed when required. The moisture level was maintained by spraying water almost twice a day, under the dry weather conditions.

Growth characteristics of different varieties are given below:

(a) Kufri Chipsona-1 (CS-1): Green, short plants with moderately thin stems, tripinnately compound leaves with smooth lamina.

(b) Kufri Chipsona-2 (CS-2): Dark green, erect and tall plants with compound leaves, undivided and rough lamina with smooth margins. Dense growth was observed

(c) Kufri Chandramukhi (KCM): Short and light green plants with thin stem, tripinnately compound leaves and plane lamina. Growth was less.

(d) Kufri Ashoka (AS): Light green plants with bushy appearance, thick stem, thin, compound leaves with pointed apex and smooth lamina and margins. The growth was dense and best among all the cultivars.

(e) Kufri Pukhraj (PR): Moderately tall, dark green plants with thick stem compound leaves with thin and large lamina and smooth margins. Apex was slightly blunt.

(f) Kufri Jyoti (KJ): Short to moderately tall, light green plants with thin stem, small to very large, shiny, compound leaves. Lamina was curved inside, with pointed apex.

(g) Cultivar Desiree (DE): Short to moderately tall, light green plants with both simple and compound leaves. Lamina was not smooth.

**Fig. 1.** FIELD CULTIVATED PLANTS OF KUFRI CHIPSONA-2(CS-2)

**Fig. 2.** FIELD CULTIVATED PLANTS OF KUFRI JYOTI (KJ)

## 4.3 HARVESTING OF THE TUBERS

The minitubers of all the potato cultivars were harvested after three and a half months of plantation. It is apparent that the minitubers from different cultivars might vary with respect to their developmental stage. However, morphologically there are differences in the shape and sizes of the minitubers where minitubers from all the varieties, were more or less spherical in shape. The minitubers were observed to be skin coloured to light brown in colour, except in the cultivar Desiree where the minitubers were reddish in colour. Essentially the tubers of all the varieties were with delicate tuber skin. The weight of most of the minitubers from all the varieties was observed to be in the range of 2 to 3 gms (data not shown). The range of the tuber weight was comparatively higher in case of CS-2. The average weight of the tubers was 1.5 to 2.0 gms. Minitubers from few potato varieties are shown in the **Fig. 3 to Fig. 6**. Harvested tubers from each cultivar were used immediately for the subsequent experiments and also used after transferring to different experimental temperature for a period of three weeks. Along with the above tubers, fresh tubers were also procured from local market (termed as MP) and used for subsequent studies.

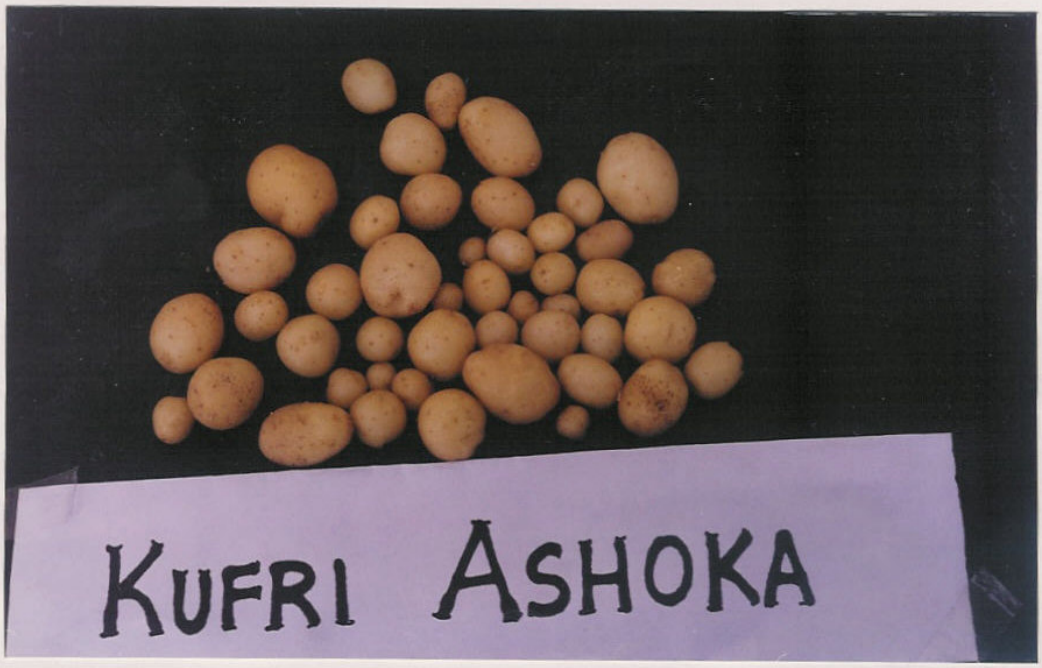


**Fig. 3.** MINITUBERS OF POTATO VARIETY KUFRI ASHOKA (AS) HARVESTED FROM THE FIELD


**Fig.4.** MINITUBERS OF POTATO VARIETY KUFRI CHIPSONA-2 (CS-2) HARVESTED FROM THE FIELD


**Fig. 5.** MINITUBERS OF POTATO VARIETY KUFRI PUKHRAJ (PR) HARVESTED FROM THE FIELD

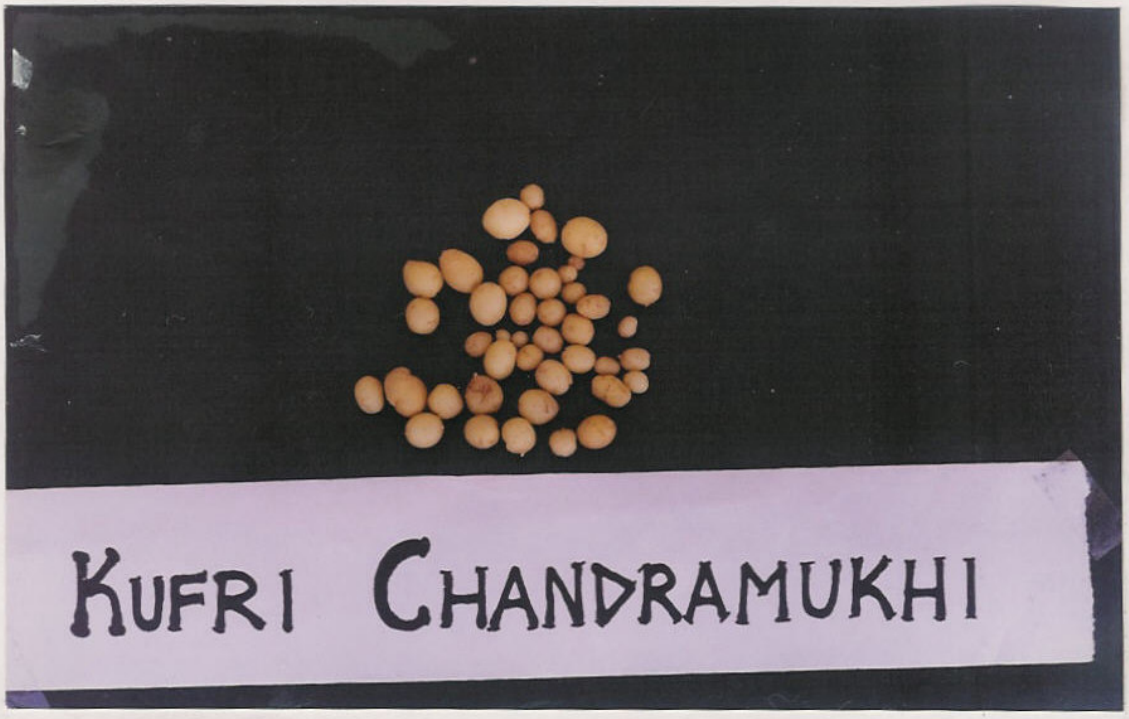


**Fig. 6.** MINITUBERS OF POTATO VARIETY KUFRI CHANDRAMUKHI (KCM) HARVESTED FROM THE FIELD

### 4.4 ISOLATION OF STARCH GRANULE AND MICROSCOPIC STUDIES

Here, freshly harvested as well as tubers stored at 4º C and 20º C for period of three weeks were taken for starch granule isolation. Homogeneous slurry was made from thin tuber slices and starch granules were recovered after extensive washings with water and acetone respectively. Finally, the starch granules were dried at 37º C for a period of 10-12 hours. The starch granules recovered were clean, white-coloured, and powdery in form.

To see the morphological characteristics, starch samples from a few varieties were stained using $I_2$-KI solution and observed under microscope. In case of Kufri Ashoka, starch granules appear to be more or less ellipsoidal in shape, not clustered but singly scattered, and irregular in size and shape **(Fig. 7).**

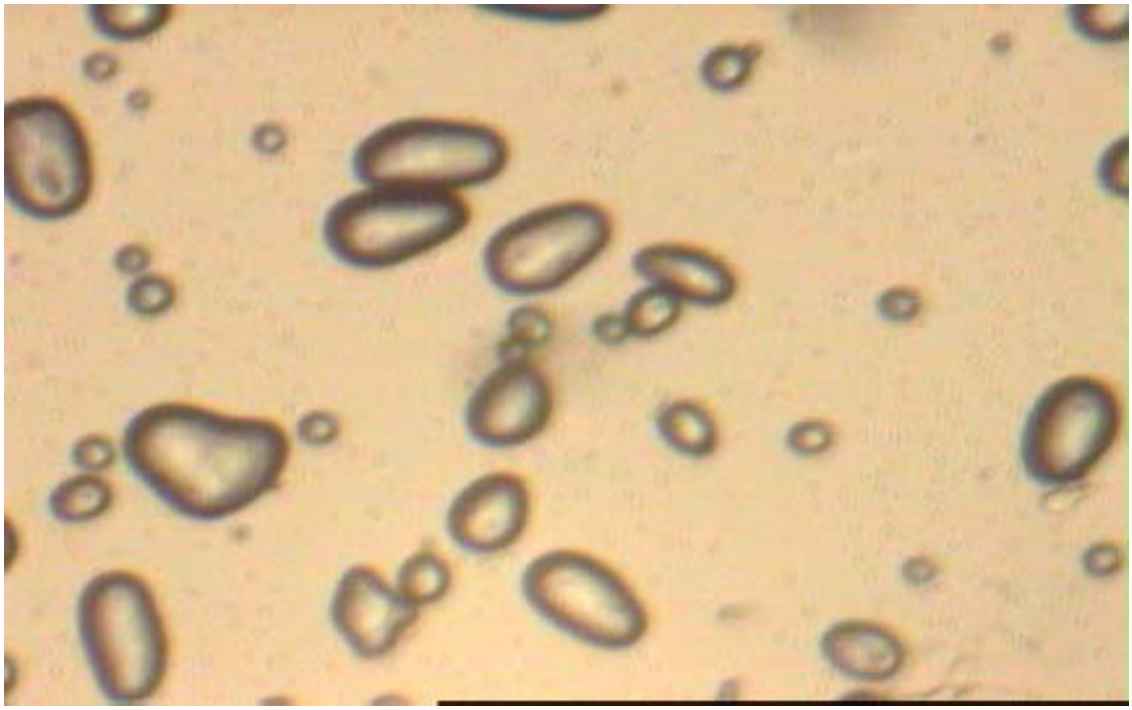

**Fig. 7** MICROSCOPIC VIEW OF STARCH GRANULES ISOLATED FROM **KUFRI ASHOKA (AS)**

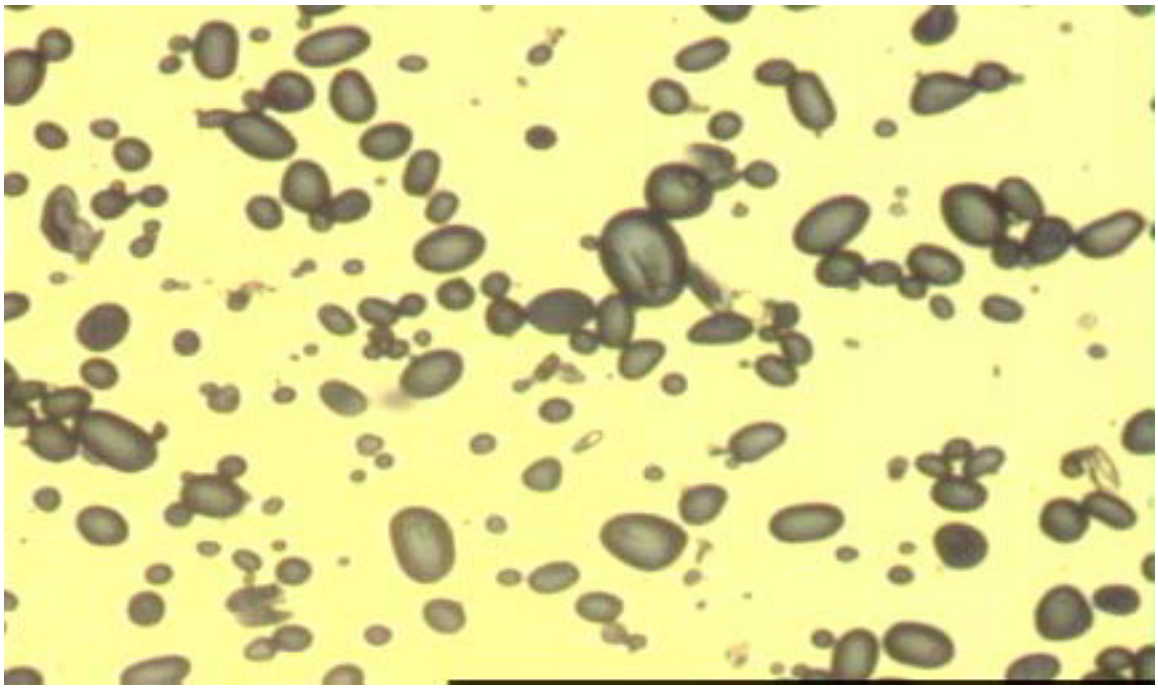

**Fig. 8** MICROSCOPIC VIEW OF STARCH GRANULES ISOLATED FROM **KUFRI CHIPSONA-1 (CS-1)**

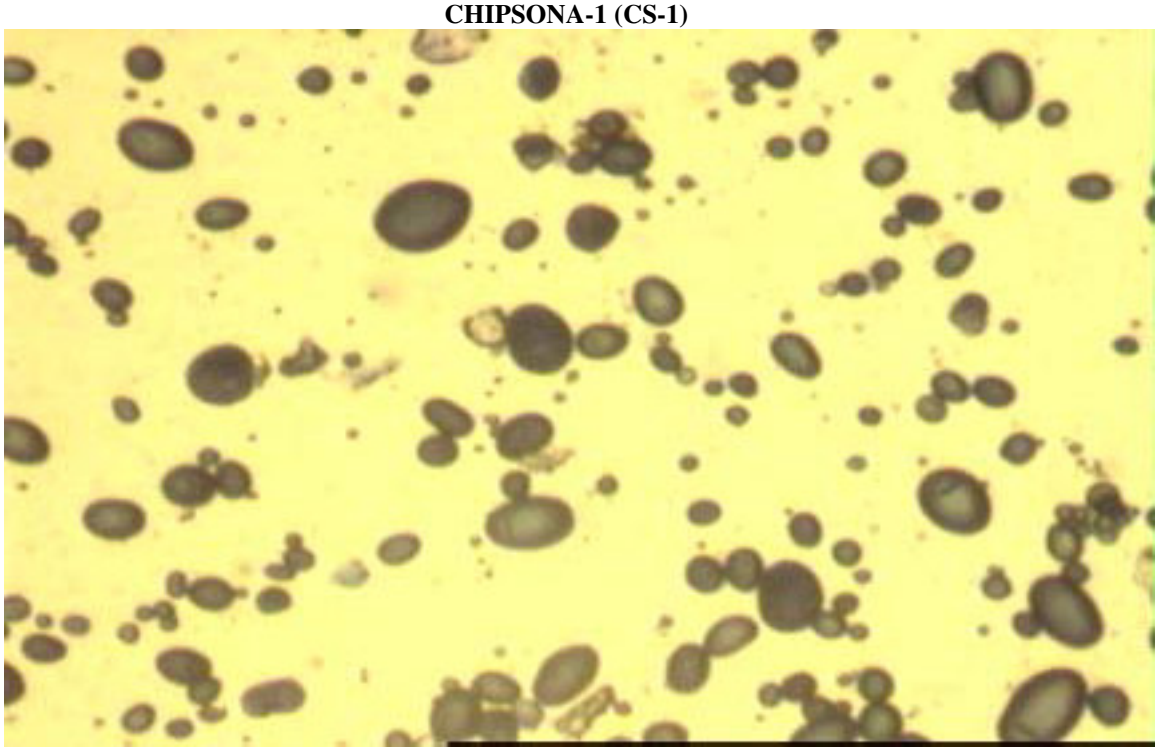

**Fig. 9** MICROSCOPIC VIEW OF STARCH GRANULES ISOLATED FROM **KUFRI CHANDRAMUKHI (KCM)**

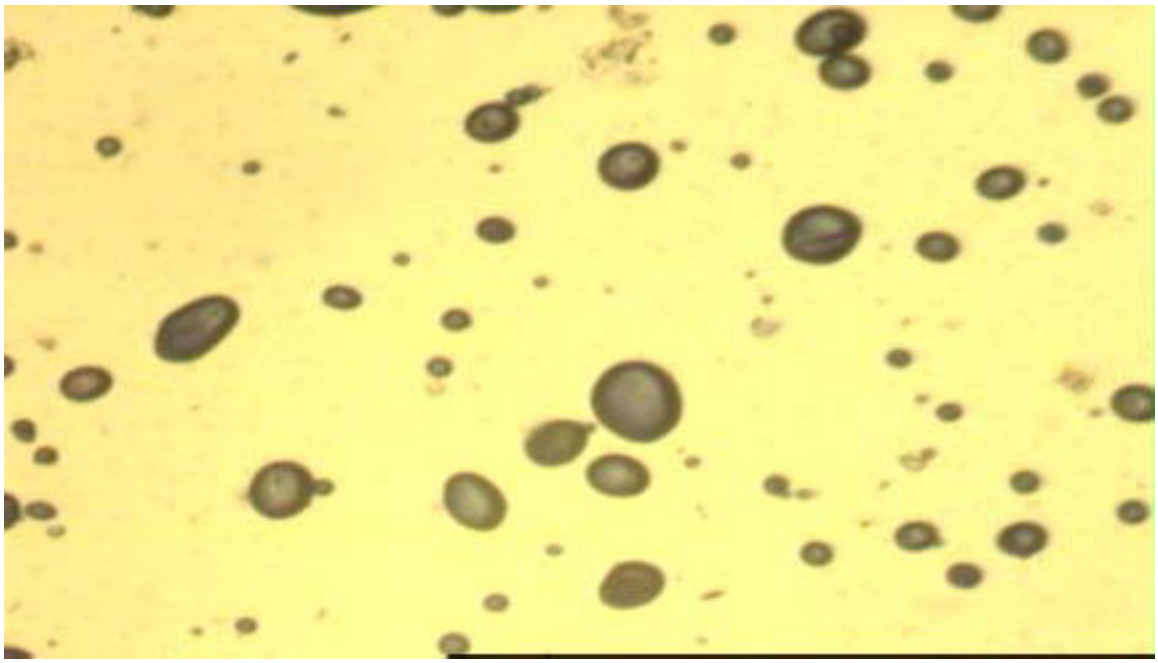

**Fig. 10** MICROSCOPIC VIEW OF STARCH GRANULES ISOLATED FROM **MARKET POTATO (MP)**

Similarly, starch granules from Kufri Chipsona-1 (CS-1) are somewhat elongated, clustered and some are seen in short chains. These granules have taken more stain as compared to granules from other varieties. **(Fig. 8)**. Similarly, starch granules from Kufri Chandramukhi (KCM) were comparatively smaller in size and irregular in shape. **(Fig. 9)**. The starch granules isolated from Market potato were mainly spherical to ovoid in shape and scattered singly **(Fig. 10)**.

### 4.4.1 QUANTIFICATION OF STARCH GRANULES IN THE TUBERS:

Weight of each starch granule preparation was taken after extensive drying at 37º C. Starch yield was calculated in gm per 100 gm of tuber weight as shown in **Table 4.1**.

In case of freshly harvested tubers, recovery of the starch granules was maximum in case of CS-2, followed by Desiree and KCM, while the minimum recovery was observed in case of MP. For tubers stored at 20º C, yield of starch granules was maximum in case of CS-1, followed by AS while the minimum recovery of starch granules was noted in PR. In case of tubers stored at 4º C, recovery of starch granules was highest in CS-1, followed by CS-2 while the minimum amount recovered from MP. Currently, CS-1 and CS-2 are considered as suitable processing varieties in terms of tuber dry matter and reducing sugar content. (*Technical Bulletin 50,*C.P.R.I *)).*

Overall the yield of starch granule from freshly harvested tubers of different varieties was in the range of 6.0 to 9.7. This may be due to genetic factors and various stages of tuber development. However, more or less suitable processing varieties like CS-1, CS-2, KCM, showing more starch yield as tuber dry matter could be directly correlated with starch content.

In case of tubers stored at 20º C, the starch granule yield is in the range of 7.2 to 10.8. If we closely analyze, there is no marked difference observed in terms of starch yield from freshly harvested tubers and that stored at 20º C In some varieties such as CS-1, AS and MP, yield of starch appears to be more as compared with freshly harvested tubers.

Possible explanations to this might be water loss during storage, being counterbalanced with respiration. Another may be due to variation with respect to stage of tuber development. It may be considered here, starch biosynthesis in the amyloplast is a developmentally regulated process.

The yield of starch granules from potato tubers stored at 4º C appeared to be lower and the range being 2.7 to 9.8. In majority of the cultivars, yield was lower as compared with freshly harvested tuber. Although it is not true for all the. Low yield is possibly due to enhanced starch degradation by cold-inducible enzymes. At the same time, we need to consider other metabolic activities also which are hitherto unknown.

TABLE 4.1. STARCH YIELD FROM VARIOUS POTATO TUBERS
(calculated in gm per 100 gm of tuber wt.)

| Varieties | **Freshly Harvested** | **Stored at 20ºC** | **Stored at 4ºC** |
|---|---|---|---|
| AS | 6.57 | 9.75 | 6.68 |
| **CS-1** | 7.93 | 10.85 | 9.86 |
| **PR** | 8.94 | 7.29 | 6.18 |
| **CS-2** | 9.73 | _ | 9.07 |
| **KCM** | 9.52 | 7.85 | 8.87 |
| **KJ** | 8.12 | _ | 5.43 |
| **DE** | 9.61 | 8.11 | 7.35 |
| **MP** | 5.90 | 8.63 | 2.74 |

## 4.5 DETERMINATION OF TOTAL PHOSPHORUS FROM STARCH GRANULES

Starch is composed of two polymers, amylose and amylopectin. The amylose molecules are essentially linear á(1•4) glucan chains whereas the amylopectin molecules are highly branched and often contain small amount of covalently bound phosphate. In addition, amylopectin, depending upon the plant organ where it is manufactured, contains different levels of phosphate monoesters. The phosphate content in the potato tubers is exceptionally high as compared with other plant storage organs. The phosphate groups are localized as monoesters at the C-6 and C-3 positions of the glucose residues in starch.

The phenomenon of cold-induced sweetening in potato tubers is known for a long time. Attempts have been made by many researchers around the world to understand the problem of cold sweetening through biochemical and molecular approaches. It is commonly believed that the above undesirable phenomenon in potato tubers is due to an increased activity of one or more cold-inducible enzymes including a few starch–degrading enzymes. However, recent experimental evidences suggest that starch phosphorylation status in potato tubers could also be correlated with the cold sweetening process. Covalently bound phosphate groups in starch molecule are considered to alter its secondary structure hence its degradability. In other words, more phosphate groups in the starch leads to more alteration in the starch structure hence its degradation.

Here attempts have been made to determine the total starch-bound phosphate in the tubers of different potato cultivars, which are more suitable to Indian agro-climatic conditions. The purpose is to know the phosphorylation status in various starch preparations. The total starch–bound phosphate i.e. organic phosphates were converted into inorganic phosphate through complete ashing procedure. Then the phosphate was determined by the Ascorbic acid-ammonium molybdate method. Starch-bound phosphate was estimated in freshly harvested tubers as well as tubers stored at 20º C and 4º C from various potato cultivars.

The data is shown in **Table 4.2** also shown in **Fig. 11** for direct comparison among cultivars.

**TABLE 4.2**: DETERMINATION OF TOTAL STARCH -BOUND PHOSPHORUS (calculated in mg per 100 gm of starch

| POTATO VARIETIES | FRESHLY HARVESTED | STORED AT 20º C | STORED AT 4º C |
|---|---|---|---|
| **AS** | 70.0 | 52.5 | 63.0 |
| **CS-1** | 87.5 | 70.0 | 105.0 |
| **CS-2** | 63.0 | _ | 77.0 |
| **PR** | 49.0 | 70.0 | 66.5 |
| **KCM** | 60.0 | 73.5 | 108.5 |
| **KJ** | 80.0 | _ | 91.0 |
| **DE** | 66.5 | 42.0 | 98.0 |
| **MP** | 90.0 | 70.0 | 98.0 |

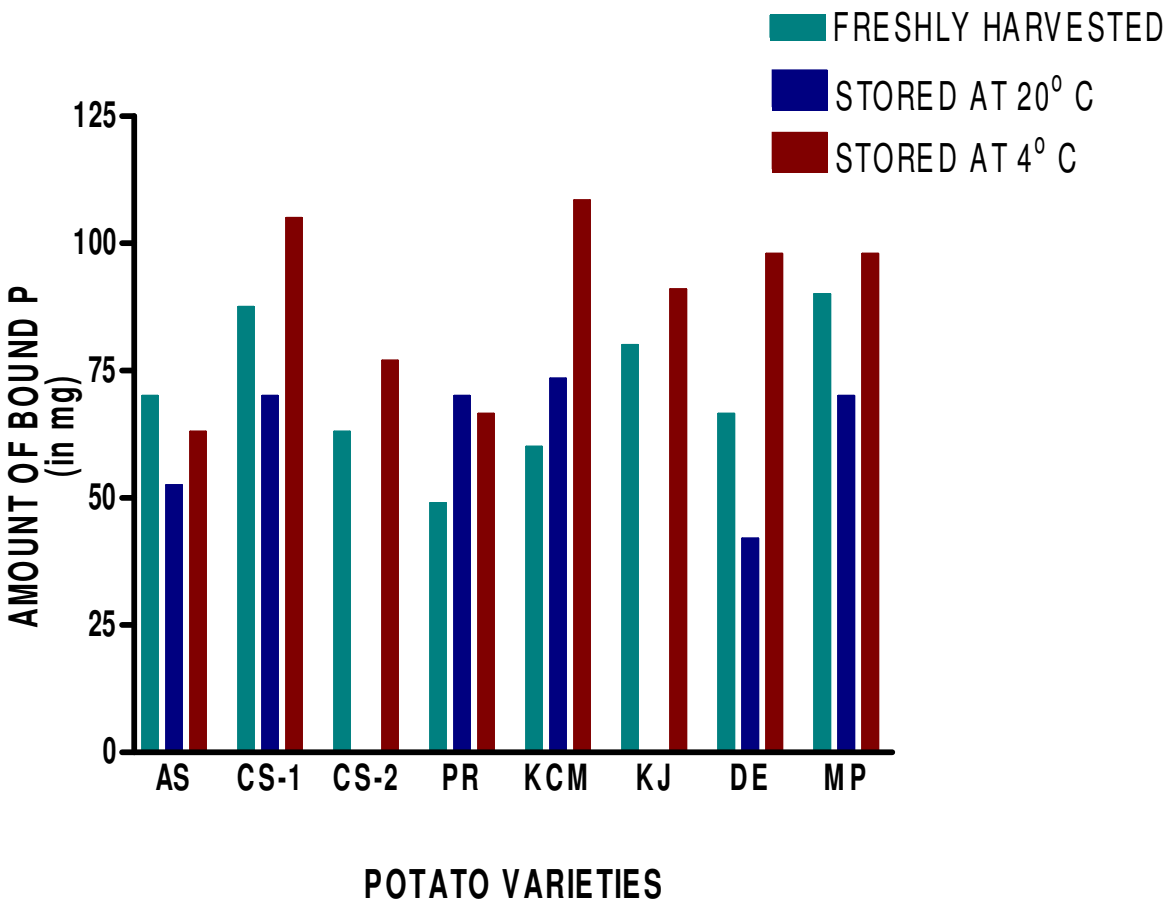


**Fig. 11.** Comparison of total phosphorus content in starch samples in potato cultivars

NOTE:The charring of a few starch samples was also carried out by making suspension in alcoholic magnesium nitrate [$Mg(NO_3)_2.6H_2O$] solution followed by assay of Pi as described earlier.The purpose was to check the sensitivity of the assay procedure. Nearly comparable data was obtained in both the ashing procedures**.**

If we closely analyse the data, there was no marked difference observed in the total starch–bound phosphate in the freshly harvested tubers and that in the tubers stored at 20º C. In case of freshly harvested tubers, the range of the starch-bound phosphate noted was nearly 65.0 mg to 80.0 mg per 100 gm starch granule. The level of phosphate seems to be higher in case of MP and CS-1. But the variety PR shows least amount of starch-bound phosphate i.e. 49.0 mg per 100 gm starch granule. In case of KJ and MP, starch from the freshly harvested tubers contains higher amount of bound phosphorous as compared with other varieties. The variation of phosphorus among reason for this might be due to genetic factors or the varietal differences .

The range of the starch-bound phosphate observed in case of tubers of various cultivars ,stored at 20º C is 50.0 mg to 70.0 mg per 100 gms of starch. But these values are almost comparable to the level of starch-bound phosphate in freshly harvested tubers. KCM shows the maximum value here i.e 73.5 mg followed by CS-1,PR and MP, all of which show 70.0 mg bound phosphate per 100 gm starch. The exotic cultivar DE showed least amount of of bound phosphate ie.42.0 mg per 100 g starch.

After storing the tubers at 4º C, for a period of three weeks, overall the amount of starch-bound phosphate slightly increased in most of the potato cultivars. Although overall increase is not that significant except CS-1 and KCM. In the latter two cases the increase is bit more on a higher side.However,the overall increase may need some attention here. Here total starch granule preparations were used for estimation of phosphorus content. It is generally believed that during tuber development the extent of starch phosphorylation also depends on

its size. In other words, size of the starch granule may affect the starch phosphorylation during tuber development. Phosphate groups preferentially bind to smaller starch granules as compared to the larger starch granules.Therefore apparent increase of phosphorus content could be due differential degradation of starch granules.

Also the presence of free phosphate was checked in the starch from tubers of different potato cultivars by suspending the starch granules in water and after some time centrifuging the suspension and estimating the supernatant for phosphate by ascorbic acid –ammonium molybdate assay. The results show that there was negligible amount of phosphate present in the starch samples from all the cultivars. In other words, it can be concluded through the above experiment that phosphorus present in the starch molecules of the potato tubers is in bound form,which can be furthre assayed by ashing procedure.

Attempts need to be made further for fractionation of the starch granules through the nylon mesh followed by gravity sedimentation of the granules, and assay of bound phosphates in various fractions of starch granules.This might give us more clear picture with respect to starch phosphorylation status. Present studies suggest that change in the status of starch phosphorylation in potato tubers is not that significant during storage at low temperature. But it needs to be understood further.

It is believed that secondary structure of starch is altered by the level of bound phosphate groups. More phosphate groups facilitate stach degradation which is one of the possible reasons of cold-induced sweetening. At the moment it is difficult to find a correlation between status of starch phosphorylation and cold sweetening in various potato cultivars (Ms Aashana Goel, personal communication ).It would be worth pursuing to know the minimum level of starch phosphorylation below which cold sweetening could be lowered. This can be achieved by inhibiting enzymes / proteins responsible for starch phosphorylation through transgenic approach. The profile related to starch

phosphorylation status made in this study would enable us to select a few cultivar varieties for further work.

## 5. FUTURE PERSPECTIVES

Most of our work has been concerned with understanding the correlation between the phosphorylation status of starch in different potato varieties, with the cold-induced sweetening in the tubers. Work done so far does not highlight other aspects which might be responsible for the cold-induced sweetening in the potato tubers. For example, it might depend upon the plant age, phase of development and maturity of the tuber tissue, varietal differences,genetic make-up of different varieties. Also, tuber dry matter is an important parameter which is responsible for good processing quality of the potato tubers and the proporation of the amount of starch studied with the tuber dry matter, along with the amount of reducing sugars present in the tubers for processed potato products,needs to be understood,to relate the granular structure, to the applications following processing.

Attempts need to be made to fractionate the starch granules and then to see the phosphorylation status of the starch ,indivisually because the phosphorylation may vary with the size of the starch granules.

A comprehensive approach has not been made so far to study the correlation between the starch phosphorylation and cold sweetening. Attempts have been made to study the phosphorylation status profile among the varieties,but this aspect needs to be explored further before reaching a consensus, to chose the varieties for transgenic approach.

The phosphate groups are located as monoesters at the C-6 (approx. 70%) and at the C-3 (approx. 30%) positions of the glucose residues. In native potato starch, 0.3-0.4% of the glucose residues in the amylopectin are phosphorylated.

Although many starch biosynthetic enzymes are known, it has never been possible to synthesize semi-crystalline glucans *in vitro*, nor has it been explained how the phosphate monoesters are incorporated into starch. Furthermore, it is not clear at the moment which enzymes are responsible for the breakdown of starch in vegetative plant organs. In order to address these open questions in starch metabolism researchers have isolated proteins bound to potato starch granules, raised antisera to these proteins and used the antisera to screen cDNA libraries for corresponding clones. One of the resulting clones, designated R1, is encoding a 160-kDa protein that is partially localized on starch granules.

The level of R1 protein present in the tissue and the biochemical mechanism by which the R1 protein phosphorylates amylopectin remains to be elucidated.

## SUMMARY

As cold-induced sweetening in potato tubers is a serious concern, a comprehensive biochemical and molecular approach needs to be undertaken to overcome such problem in the cultivars suitable to our agro-climatic conditions. Currently, phosphorylation status of starch in potato tubers is believed to play a crucial role in the above process as phosphate groups attached to glucose residues at C-6 and C-3 positions alter overall secondary structure of starch hence its degradability at low temperature. We need to have an insight to find correlation between starch phosphorylation and cold sweetening process.

The present dissertation work deals with the studies on several potato cultivars namely CS-1, CS-2, KCM, AS, KJ, PR, cultivar Desiree, which were routinely micropropagated in our laboratory. After proper hardening and acclimatization, the potato plantlets of the above mentioned varieties were cultivated in the field for a period of three and a half months (mid of November to end of February). Minitubers were then harvested from each variety. The minitubers were used immediately after harvest as well as after storage at 20º C and 4º C for a period of three weeks for subsequent experiments. Isolation of starch granules was carried out from various tuber samples. Starch granule preparations from various cultivars were studied under microscope after iodine staining to see the differences with respect to size, shape and distribution. The starch granules were also quantified in gm per 100 gm of tuber weight. Attempts have been made to estimate the starch-bound phosphorus from freshly harvested tubers and that stored at 20º C and 4º C of the above cultivars using very sensitive ascorbic acid-ammonium molybdate assay method. The level of free Pi was also checked in the starch granules. The purpose was to see the effect of low temperature storage on total phosphorus content in starch.

A profile of the phosphorylation status of starch from various potato cultivars made in this study would help us to understand more about the process of cold sweetening and also to select a few varieties for further transgenic work.

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