MATERIAL AND METHODS

METHODS OF PLANTING

'Seed tubers' of various genotypes of *Solanum tuberosum* ssp. *tuberosum* were planted in the Botanical garden of the Department of Botany, Faculty of Science and garden of R.E.I. building of Dayalbagh Educational Institute, Dayalbagh Agra. The genotypes used were MST, Kufri Jyoti and kufri Badshah. They were sown from September 25, to September 30, during the years 1994 and 1995 (Fig. 1).  

BRICK PLANTING

The seed tubers were planted in an area of 5.40x2.50 sq. metre and 5.50x3.45 sq. meter on bricks. This method was developed by Thijn (1954) and used by other workers (Abdulwaheb and Miller, 1963; Juneja, 1984; 1988) in which the tubers were placed over a half brick and planted in the soil pit (Fig. 1). For planting on bricks, pits were dug in each row 20cm apart to a depth of 15cm. Ten grams of Benzene hexachloride (BHC) powder was sprinkled in the pit along with cowdung manure mixed with the soil and half a brick was placed in each pit. This method promotes rooting and suppresses the formation of stolons and tuberization.  

The first irrigation was given on tenth day in the furrow inbetween the rows. Tubers sprouted in the second week of October, that is between October 12 and 14.  

RAISING TOMATO PLANTS TO BE USED AS ROOT STOCKS FOR GRAFTING POTATO SCIONS

The areas of field on which tomato plants were grown as root stock were 5.40x3.40, 4.30x2.50 and 5.50x3.45 sq. metres.  

Seedlings of tomato were raised by sowing seeds between September 18 to 20, during both the years, 1994 and 1995. They were ready for transplanting by first week of October.  

Seedlings were transplanted in experimental field of
the Botanical Garden, Faculty of Science as well as in the garden of R.E.I. Building, Dayalbagh Educational Institute, Dayalbagh in the vicinity of potato plantations.

These were transplanted in rows, keeping space of 30cm between individual rows and keeping each plant 30cm apart.

The garden beds were prepared for transplanting tomato seedlings by dividing them as plots of 60 cm. area lined by bricks. This was done to facilitate convenient grafting operation and observation of each plant and to mark out for individual genotype at the stage of grafting.

After 20 days, when the tomato plants got established and had attained proper stage with 5 to 7 leaves and height of 8 to 10 cm. they were ready to be grafted.

**GRAFTING TECHNIQUE**

Grafts were made between shoot tips of potato with three leaves as scions (Fig. 2A) and tomato plants as a root stock. For this potato shoot tips were excised and were dipped in a beaker of tap water.

The tomato stocks were prepared by decapitating the tip of shoot with a sharp blade leaving the lower portion with two or three nodes with leaves intact. The cut stump and the lower portion of the shoot were washed well by gently rubbing the surface with water. A median vertical incision of 2 to 2.5 cm. long was made through the cut stump and was kept wet by dripping water on it to avoid dehydration.

In each of the shoot tips (scions) of potato 1.5 to 2.0 cm. long wedge was made at the base by two slant vertical cuts to a thin edge below (Fig. 2B).

The wedge of potato scion was inserted straight into the cut incision made in tomato stock plant and the grafted region was fastened with a thin and narrow polyethylene strip (Fig. 2C-E).
The plants on which grafts made were covered large polyethylene bags to protect them against the chill cold nights. The bags were removed around 10 O'clock in the morning daily. Covering and exposing of grafted plants were continued until the grafts joined and became established. This is achieved when the grafts have put forth 2 or 3 new leaves at the top of potato scion. Grafts were united completely when few more leaves have grown on the potato shoot. At this stage polyethylene strips were also ready to be untied (Fig. 3A).

The leaves of tomato stock were also removed one by one (time to time) as the leaves of potato scion increased in number leaving behind a leafless root stock of tomato (Fig. 3B, C) on which is supported the grafted potato plant. Grafted potato plant gets established and grows lavishly.

**POLLINATION**

The grafted plants growing in an area of 3.20 x 2.50 meters having three rows of 10, 12 and 13 plants were left for natural pollination. The fruits were harvested 15, 18, 21, 24, 27, 30, 33, 36 and 40 days after pollination which were marked from the stage of anthesis of floral bud.

Besides, the buds and flowers which were allowed to be naturally pollinated in the field (grown in plots in an area of 3.50 x 2.80 meters) have been repeatedly pollinated by hand. The grafted plants growing were in four rows of 10, 10, 12 and 13 in number.

A detailed work of repeated hand pollinations revealed satisfactory result (Upadhya et al. 1984; Juneja 1988) on increased number and better quality of seed production of potato.

As established the stigma of potato flower remains receptive for 30 to 40 hours and is able to receive and support germination of pollen.

Hand pollinations were performed repeatedly four times on the same stigma which may seem to be more useful over
three hand pollinations to bring about successful fertilization and formation of large number of better quality of fruits and seeds. The buds and flowers were emasculated and bagged after hand pollinations. Some of the remaining flowers were emasculated and hand pollinated but left unbagged.

The pollinations were therefore carried on, beginning from evening at 16.30 hours (at 0 hours) with first pollination followed by second pollination at 7.30 hours next morning (15 hours later). The third pollination commenced at 16.30 hours (next 24 hours) and the fourth pollination was performed on morning of second day at 7.30 hours. (39 hours later).

**POLLEN VIABILITY TEST**

2,3,5 - triphenyl tetrazolium chloride (TTC) -

Prepared a 0.1 per cent Triphenyl Tetrazolium chloride and stored in a brown glass bottle. Poured 10 ml of TTC solution in petriplate and sprinkled pollen grains and kept in the dark for 2 to 3 hours at temperature of 30°C after closing. The pollen grains were then examined under the binocular microscope.

**Sucrose Test**-

A semi-solid medium of 0.8 per cent agar with four different concentrations of sucrose (30; 50; 70 and 90 per cent) was prepared.

This was then autoclaved at 120°C temperature and 15 lbs pressure for 15 minutes. The four sets of 30 per cent; 50 per cent; 70 per cent and 90 per cent sucrose respectively were poured in sterilized petriplates by dividing the solution into two for each concentration. The medium was then set in the room temperature.

Pollen grains from one mature anthers were sprinkled on each petriplate medium and kept aside for direct observation.

The sprinkled pollen grains on each were counted initially.
and thereafter, at an interval of 30 minutes. The germinated pollen were then counted at particular intervals.

(A) QUALITATIVE MICROCHEMICAL TESTS FOR RESERVED FOOD MATERIAL

1. TESTS FOR REDUCING SUGARS

This requires the use of some reagents as follows -

Fehling's solution. It was prepared just before use by mixing equal volumes of the following reagents.

Fehling's A

It was prepared by dissolving 35 grams CuSO₄·7H₂O in distilled water made up to 500 ml.

Fehling's B

It was prepared by dissolving 120 grams of KOH and 137 grams of sodium potassium tartrate in distilled water and volume made up to 500 ml.

The following parts of potato plant were taken: underground dormant tuber, sprouted tuber, aerial tuber and calyx, corolla, anthers, pollen grains, stigma, ovary, unripe and ripe fruits as vegetative parts and as reproductive parts respectively.

Each one of the potato plant parts mentioned above was crushed and made into a crude extract separately using homogenizer.

To 5 ml of this extract equal amount of Fehling's A and B solutions (2.5 ml of each) were added in a test tube. They were mixed well by shaking and then heated over a flame and cooled. Different range of yellow to rustish orange coloured precipitate appeared according to the extracts from various parts used.

2. TEST FOR STARCH

The reagent IKI solution was used to detect starch. The following plant parts were taken-underground dormant
tuber, sprouted underground tuber and aerial tuber as vegetative parts and floral parts as calyx and corolla lobes, anthers, pollen grains, ovary and stigma. Each one of the parts mentioned above was crushed and made into a crude extract separately.

Taking one at a time in a test tube 5 ml of each of the extract and 5 drops of the reagent IKI solution was added. This resulted in production of blue-black colouration.

3. TEST FOR PECTIN

The reagent methylene blue was used to detect pectin.

The following plant parts were taken - stigma, style, pollen grains, pollen tubes, unripe, ripe fruit and seed.

Each one of the potato plant parts mentioned above was crushed and made into a crude extract separately using homogenizer.

To 5 ml of this extract equal amount of methylene blue were added in a test tube. They were mixed well by shaking. This resulted in rendering blue to light blue colouration to the cell surface.

4. TEST FOR FATS

The reagent Sudan III was used to detect fat. The vegetative and reproductive plant parts were used as underground dormant tuber, sprouted tuber, aerial tuber, unripe and ripe fruits. A crude extract for each of the parts was made by crushing the material with distilled water in the homogenizer.

To 5 ml of material extract, 5 ml of Sudan III was added. It was mixed and shaken well. Orange to red colour appeared in the oil globules that released from the cells.

5. TEST FOR PROTEINS

The Millon's reagent and Biuret was used to detect proteins.

The following plant part were taken underground dormant
tuber, sprouted underground tuber, aerial tuber as vegetative parts and reproductive parts as calyx, corolla, anther, pollen grain, ovary, stigma, style, unripe and ripe fruit and seeds.

Each one of the potato plant parts mentioned above was crushed and made into a crude extract separately using homogenizer.

Taking one at a time in a test tube 5 ml of each the extract and 6-8 drops of the Millon’s reagent results in coagulated material that turn pink to light pink. This observation revealed the presence of proteins.

(B) QUANTITATIVE ANALYSIS OF STORED FOOD MATERIALS AS REDUCING SUGARS AND PROTEINS

1. THE QUANTITATIVE ANALYSIS OF REDUCING SUGARS

The test was performed by Fehling A and B reagents. The plant parts used were underground tuber, aerial tuber, unripe and ripe fruits and seeds. The plant material was homogenized with distilled water and an extract was prepared from each part.

Equal volume of extract and the Fehling A and B solutions (5 ml) each were poured into a test tube. They were mixed, heated and cooled. An orangish rustish red colouration developed. The content was centrifuged and the precipitate was procured.

The precipitate was put into a crucible. After weighing it was kept in muffle furnace at 100 to 120°C for 3 to 4 hours. After cooling the precipitate content of the crucible was weighed again. This is dry weight. The percentage of precipitate was calculated, which gives the amount of reducing sugars.

2. QUANTITATIVE ANALYSIS OF TOTAL PROTEINS

The test was performed by Millon’s reagent. The plant parts used were underground tuber, aerial tuber, unripe and ripe fruits and seeds. The plant material was homogenized with
distilled water and an extract was prepared from each part.

Equal volumes of extract and the Millon's reagent (5ml each) were poured into a test tube. They were mixed and heated and cooled. It gets coagulated to pink colour. The residue was procured by using centrifuge.

The residue was put into a crucible. After weighing it was kept in muffle furnace at 100 to 120°C for 3 to 4 hours. After cooling the residue content of the crucible was weighed. This is the dry weight. On basis of this dry weight, percentage was calculated. This gives the total proteins in a particular potato plant part.

ESTIMATION

ESTIMATION OF GLUCOSE

This required the use of some reagents as follows:

**Reagent A** - Picric acid- Thirty six grams of picric acid was dissolved in 400 ml of distilled water with 500 ml of 1 per cent NaOH making upto final volume of 1 litre.

**Reagent B** - Anhydrous sodium carbonate solution- It was prepared by dissolving 16.5 gm in 100 ml of distilled water to make 16.5 per cent.

To make 0.04 per cent Standard glucose solution - 2 mg of glucose was dissolved in 500 ml of distilled water.

The above solution was pipetted and poured into a series of test tubes as 0.0; 0.5; 1.0; 1.5; 2.0; 2.5 and 3.0 ml. Test tube with 0.0 concentration was considered as control and functioned as zero reading for estimation of glucose. To each of the test tubes 4 ml of reagent 'A' and 1 ml of reagent 'B' were added and each was made to the final volume of 10 ml with distilled water.

The whole set of series was kept in a water bath at 90°C for 15 minutes. The content of the test tubes rendered range of dark yellow, mustard, brown, dark brown, and deep brown colours. This colouration was given by picric acid
reagent.

The following plant parts were taken: underground tuber, aerial tuber, sprouted tuber, calyx, corolla, pollen, anther, style, unripe and ripe fruits. All these parts were crushed and homogenized to prepare extract individually and poured into a test tubes as 1 ml each a green filter was used as the range of colour was from yellow to brown. Recorded the readings as optical density and percentage transmittance of each test solution using photocolorimeter (Lindsay, 1973).

ESTIMATION OF STARCH

The following solutions were required. They were prepared as follows.

Solution A - Prepared a standard solution of starch powder at a concentration of 1 per cent by dissolving 1 gm in 100 ml of distilled water.

Solution B - Iodine reagent (IKI solution).

The standard starch solution was poured into series of test tubes for preparing various concentrations as 0.0; 0.5; 1.0; 1.5; 2.0; 2.5 and 3.0 ml. To each of the test tubes 4 ml of distilled water and exactly 1 ml of iodine reagent were added. Final volume was made to 10 ml with distilled water in each.

Test tube with 0.0 concentration was considered as control and it functioned as zero reading of the photocolorimeter. A red filter was used against the test colouration which was in the range of blue to blue black. Optical densities and percentage transmittance were recorded for standard graph.

For estimation of starch the following plant parts were used—underground tuber, sprouted tuber, aerial tuber, calyx, corolla, pollen grains, stigma style, unripe and ripe fruit and seed.

These were crushed and homogenized individually into
an extract and poured into a series of test tubes as
0.5;1.0;1.5;2.0;2.5 and 3.0 ml to match with various
concentration of the series of standard solution.

A red filter was used as usual and recorded the
optical density (O.D) and percentage transmittance. The readings
were used to plot graph for quantitative estimation of starch.

LOWRY'S METHOD FOR PROTEIN ESTIMATION

For using Lowry's method the following solutions were
required. They were prepared as follows:

Reagent A - 2 per cent Sodium carbonate is prepared
in 0.1 N NaOH solution.

Reagent B - It is prepared by mixing 0.5 per cent
Copper sulphate solution in 1 per cent Sodium potassium
tartrate solution.

Both were prepared separately in distilled water before
mixing.

Reagent C - This was prepared fresh by mixing 50 ml
of reagent A and 1 ml of reagent B to give an alkaline
mixture.

Reagent D - Trichloroacetic acid (TCA): This was pre-
pared by dissolving 5 gm of trichloroacetic acid in 100 ml
of distilled water.

Reagent E - 0.1 N Sodium hydroxide solution was
prepared by titrating it against 0.1 N Oxalic acid solution.

Reagent F - Standard Protein solution; This was prepared
by dissolving 5 mg of Bovine serum albumin in 25 ml of
distilled water.

Reagent G - Folin's Reagent

This was prepared by weighing 100 g Sodium tungstate,
25 g Sodium molybdate and dissolving them in 500 ml of
distilled water. To this 50 ml of 85 per cent phosphoric acid
and 100 ml of concentrated Hydrochloric acid was added. The mixture was refluxed gently for about 10 hours with a condenser. This helps to remove excess bromine. After cooling the volume is made up to 1 litre and filtered, if necessary. The filtrate should not have any greenish tinge. If it has, it is boiled with bromine once more. This is the stock reagent and is diluted with equal volume of water just before use. This is also commercially available and has to be diluted as above and used.

To prepare a set of different concentrations 0.5; 1; 1.5; 2.0; and 2.5 ml of the test solution (Reagent F) was poured in a series of test tubes using pipette and was made up to the total volume of 4 ml with addition of distilled water. The control test tube contained 4 ml of distilled water only. To each tube 5.5 ml of the alkaline mixture (Reagent C) was poured, mixed well and allowed to stand at room temperature for 10 to 15 minutes. To each test tube 0.5 ml of the reagent G was poured. The test tubes were left as such for 30 minutes. A range of blue colour developed according to the concentrations in the test tubes. It was measured at a wavelength of 650 nm using U.V. spectrophotometer. The standard graph was revealed between different concentrations and their absorbance.

The above reagents for Lowry's method were used for estimation of protein in various parts of potato plant such as underground tuber, aerial tuber, calyx, corolla lobes, anther pollen grains, ovary, stigma and style. They were separately crushed and homogenized to obtain extracts of each.

In a test tube 1 ml of extract from a particular part was taken to which 1 ml of 5 per cent TCA was added and mixed gently. To this 1 ml of 0.1 N NaOH and 5.5 ml of alkaline Copper sulphate solution (reagent C) were added. The mixture
was made up to 10 ml by adding distilled water and was kept aside. After 10 to 15 minutes 0.5 ml of 1 N Folin reagent was added and allowed to incubate for 30 minutes. A blue colour developed.

SEPARATION OF AMINO ACIDS BY CHROMATOGRAPHY
TECHNIQUE

PAPER CHROMATOGRAPHY

This method is used for the detection and separation of amino acids.

The pure amino acids used included Aliphatic amino acids- glycine; valine; leucine; isoleucine; Hydroxylic amino acids- Serine and threonine; sulphur containing amino acids- cystine and methionine; Acidic amino acids- asparagine; asparatic acid and glutamine; Basic amino acids- Lysine; arginine; Aromatic and Heterocyclic amino acids- phenylalanine; tyrosine; tryptophan and histidine and an imino acid- proline. These were prepared as 20 per cent solution in distilled water and stored in the labelled dropping bottles in the refrigerator at 7° - 15°C.

Different materials under study were excised and crushed with distilled water with the help of homogenizer and was made into a fine paste.

Whatman filter paper no. 1 was used. It was neatly cut into rectangular pieces of 26cm x 22cm or 10.5cm x 5.0cm according to the sizes of the chromatography chambers. A base line was then drawn on the chromatography paper at a distance of 1.5 cm from the base. Points were marked at equal distances on it. Each of such marks was used for loading the spot of individual known amino acid. The loading of same amino acids was repeatedly done on the initial marks. The process was repeated 8 to 10 times until the spots were sufficiently concentrated. They were dried each time.

The solvent mixture was prepared in the ratio 4:1:5
by mixing 40 volumes of n-butanol; 10 volumes of glacial acetic acid and 50 volumes of distilled water. It was poured into the chromatographic chamber. The chromatographic paper that was previously loaded with amino acid spots was slipped and hung inside the chamber with the help of piece of string tied on top and supported by clips. The lower 3mm edge of paper was just dipped into the solvent mixture taking care that the spots on the base line do not come in contact with it.

Vaseline was smeared on the underside of the lid and the edges of the chamber. The chamber was then covered by the lid to make it air tight. The experimental set up was kept for two hours. By this time there was no more upward migration of the solvent mixture and the level became constant.

The chromatography paper was then removed from the chamber and the solvent mixture line was marked faintly by pencil. The chromatogram was dried thoroughly in air. A solution of 0.1 per cent Ninhydrin in Acetone was prepared fresh and the dried chromatogram was sprayed with it. It was kept for drying in air or inside an oven at a temperature of 60°C for few minutes. The coloured spots of the respective amino acids started appearing at their migration sites on the chromatogram.

The Rf values for each were calculated. The ratio of the distance travelled by a component to that travelled by the solvent front both were measured from the marked point of the mixture is called the resolution front (Rf) value for that component.

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Rf = \frac{\text{Distance from origin run by the component}}{\text{Distance from origin run by the solvent}}
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HISTOCHEMICAL STUDY
COLLECTION AND FIXATION OF MATERIAL OF POTATO

The plant material as buds, flowers, fruits and their parts at various stages of development were fixed and preserved. They were fixed in different fixatives as FAA and Carnoy's fluid.

FIXATION
A PREPARATION OF FIXATIVE

1. FAA - 70 per cent ethyl alcohol (ethanol) was prepared and to 90 ml of this 70 per cent alcohol, 5 ml of glacial acetic acid and 5 ml of formaline were added (Johansen, 1940; Gomori, 1952; Sass, 1958; Hopwood, 1969; Pearse, 1972; Bhandari, 1996) making the ratio as 18:1:1.

2. Carnoy's fixative - It is made by mixing 6 parts of absolute alcohol and 3 parts chloroform and 1 part glacial acetic acid. (Sass, 1958; Jensen, 1962; O'Brien and McCully, 1981).

DEHYDRATION, INFILTRATION AND EMBEDDING

The material fixed in FAA and Carnoy's fluid is preserved in 70 per cent ethanol. Clearing and dehydration of the fixed materials was done through ethanol and xylene series. They were infiltrated and embedded in Merck's paraffin wax.

DEHYDRATION

It is a process that removes water from the fixed and hardened tissues.

The dehydration reagent is absolute ethyl alcohol. A close series of dilutions of ethyl alcohol in water at concentration of 30 per cent; 50 per cent; 70 per cent; 90 per cent; Absolute (A) and Absolute (B) were prepared and poured in tightly corked wide mouthed bottles and the preserved material was passed through it. This was followed by series consisting of combination of xylene and ethyl alcohol in the ratio of 2:1 and 1:1; pure xylene (A) and (B). This is called passing series. The materials were put into the passing tubes
and then dipped in passing series in the ascending order of alcohol and xylene one by one at intervals of 24 hours each for proper dehydration.

**INfiltration**

Infiltration consists in first dissolving the paraffin in the solvent containing the dehydrated material by gradually increasing the concentration of paraffin and decreasing the concentration of solvent.

The paraffin was kept for melting in a beaker in an oven at a temperature of 80° to 90°c. The materials in the vial of xylene gets infiltrated by paraffin and xylene mixture solvent as melted paraffin (about 1/4 volume of xylene) which was added into the vial. It was added and decanted time to time until it is finally saturated with paraffin (Johansen, 1940; Sass 1958). Each of these changes were given after 18 to 24 hours and the vials were kept in the oven at 60° to 80°c in the initial stages of changes as well as until the whole of solvent xylene is evaporated. The process was continued with pure paraffin for 48 hours more to complete infiltration. It was then kept at room temperature and covered well to avoid entry of dust particles and moisture. This material is then ready for embedding.

**Embedding**

Embedding was done in pure paraffin wax for which small embedding trays were used. The "trays" which were made by porcellin or stiff paper (Johansen, 1940; Sass, 1958). The embedding tray was smeared with glycerin. A brass tripod with a gas flame below was placed nearby the oven for heating and warming the needle and forceps used for arranging the material in the melting paraffin wax. The vial was removed from the oven and the material together with the melted paraffin was quickly poured into the glycerine smeared tray. With a hot needle or tips of forceps the pieces of
material were disposed into an orderly arrangement with proper spacing between them.

The tray was moved without disturbing the material and was transferred to a large tray of cold water. On cooling the block was removed from the embedding tray and floated in water. Small blocks were cut from the large block with one piece of material in each.

Procured some wooden blocks for mounting. The base of the paraffin block with the embedded material was melted by hot scalpel and fastened on to one of the faces of the wooden block.

MICROTOME SECTIONING OF THE PARAFFIN EMBEDDED MATERIAL

Serial sections were obtained using rotary microtome in which razor remains stationary and the paraffine embedded material moves up and down past the cutting edge of the razor. This resulted in getting successive sections that remain attached to each other forming a paraffin ribbon. The sections were cut between 10 and 12 microns or between 15 to 20 microns by adjusting the thickness using the knob of the regulator. These sections were thus kept in serial order on a thick sheet of paper.

PREPARATION OF SLIDES

The slides to be used were cleaned by dipping them in nitric acid overheight and thereafter washed with running tap water before use. This ensures thorough cleaning.

The ribbon was cut to equal smaller pieces so that they occupy 3/4th space on the slides. They were arranged in 1, 2, 3 or 4 horizontal rows.

For fastening the paraffin ribbons with sectioned material on the slide a dilute adhesive with a disinfectant was used. For this purpose two or three drop of gum arabic and few crystals of potassium dichromate were put and dissolved in a dropping bottle with water.
Two or three drops of this adhesive fluid was poured on to the slide and smeared into a thin film. The horizontally cut strips of ribbons were floated and the slide was kept on a slightly heated hot plate. This helps in straightening the wrinkles of the paraffin ribbon with material and affixing them firmly to the slide. The slides prepared this way were kept slant longitudinally to remove extra adhesive fluid. These were kept for 7-15 days for drying in a slide box before staining processes.

STAINING PARAFFIN SECTIONS

Paraffin sections affixed to slides were stained and processed by immersing them in the reagents using vertical coplin jars. These jars have grooves that hold the slides in a vertical position. Staining jars were cleaned and labelled.

The staining series consists of 'down' and 'up' series. The process of staining is done in a gradient sequence (see chart for down series) beginning with dissolving paraffin by pouring pure xylene (A) in the jar with the slides. One after the other with regular intervals of time which may be shortened depending upon the condition of the section. Transfers should be made fast to avoid drying of slides. This was kept for 3 to 5 hours, and then it was changed by pure xylene (B) and kept for 1.5 to 2 hours. The changes were given in a sequence (chart) by decanting the previous solution and pouring the next. These slides were then kept in the mixture of xylene and ethanol in the ratios 2:1 and 1:1 for 15 minutes to 2 hours in each. When changed to pure ethanol (A) and (B) they were retained and kept for another 2 hours each time. This was followed by 90 per cent; 70 per cent; 50 per cent and 30 per cent ethanol for 10 to 15 minutes each.

These slides were then thoroughly washed in running tap water and stained with various stains for different histochemical tests.
DOWN SERIES
Paraffin sections

Xylene A (100%)

Xylene B (100%)

Xylene: ethanol
2:1

Xylene: ethanol
1:1

Ethanol A
(100%)

Ethanol B
(100%)

Ethanol
(90%)

Ethanol
(70%)

Ethanol
(50%)

Ethanol
(30%)

Thorough washing in water

Stain

[Different Stains and Procedures Used for Particular Test]
TYPES OF STAINS

Various types of stains were used for histochemical and electrophoretic studies.

1. Stains used for Histochemical studies:
   (a) Mercuric bromo-phenol blue
   (b) Coomassie brilliant blue
   (c) Periodic acid and Schiff's reagent (PAS)
   (d) IKI solution
   (e) Sudan black and Neutral red
   (f) Orange G and Aniline blue
   (g) Methylene blue
   (h) Nitrous acid test.

2. Stains used for Electrophoresis:
   (a) Coomassie brilliant blue
   (b) Sudan black
   (c) Sudan III
   (d) Schiff's reagent
   (e) Methylene blue (in buffer)
   (f) Lactophenol blue
   (g) Benzidine

PREPARATION OF DIFFERENT REAGENT AND STAINS FOR HISTOCHEMICAL STUDIES AND ELECTROPHORESIS

(A). Mercuric bromo phenol blue

The solution consists of 1 per cent HgCl₂ and 0.05 per cent bromophenol blue in 2 per cent aqueous Acetic acid, and was prepared and stored in a coloured bottle in the refrigerator. This renders blue colour for proteins.

(B). Coomassie brilliant blue

It was prepared by taking 0.25 per cent W/V Coomassie brilliant blue R 250 in 7 per cent aqueous Acetic acid. For removing excess stain 5 per cent V/V acetic acid was prepared separately. This stains total
proteins blue in colour.

(C). Periodic acid and Schiff's reagent (PAS)

(a) Periodic acid was prepared by taking 0.4 g and dissolving it in 35 ml of 95 per cent ethanol and mixing it in already prepared 5 ml of Sodium acetate (2.72% aqueous solution) solution and making it to 50 ml by adding distilled water. It gives colourless solution. This solution was kept in cold temperature and was discarded if it turns brown in colour.

(b) Schiff reagent was prepared by dissolving 1.0 g of fuchsin (basic) in 100 ml of boiling distilled water and cooling it to 50°C and then filtered. To this 2.0 g of sodium metabisulphite and 20 ml of 1N Hydrochloric acid were added and was then tightly stoppered and stored overnight in the dark at room temperature. Next morning 0.3 g of charcoal was added and thoroughly shaken for 1 minute and then filtered. It gives test for polysaccharides by rendering magenta and purplish red colour.

(D) IKI solution

Potassium iodide (2.0 g) was dissolved in an extremely small amount of distilled water. To this solution 1.0 g of iodine crystals was added and then made into 1.0 litre by adding distilled water. It gives test for starch rendering blue black or pulp stain.

(E) Sudan black and Neutral red- Saturated solution of sudan black is prepared by dissolve it in 100 per cent ethanol. Neutral red is prepared as 0.1 per cent in distilled water.

(F) Orange G-aniline blue

It is prepared by dissolving 2.0 g of Orange G and 0.5 mg of aniline blue in 100 ml of 0.1 M citrate buffer of pH 3.0. This stains phospholipids orangish.

(G) Methylene blue

It is prepared as 0.02 per cent W/V in citrate buffer of pH 3.5. It give blue colour for total nucleic acid in electrophoresis and nucleic acid (RNA) for histochemistry.

(H) Nitrous acid test

It is prepared using 10 per cent aqueous sodium nitrate, Acetic acid and 20 per cent Urea. It gives reaction with phenols.
(I) Sudan III

The stain Sudan III was prepared by taking 0.1 g and dissolved in 50 ml of acetone. After dissolving 50 ml of 70 per cent ethanol was added and stored in a tightly stoppered bottle in the dark. It was used after filtering. It renders orangish red colour for total lipids.

(J) Lectophenol blue

It was prepared in two steps.

(a) Aniline blue was made by dissolving 0.05 g in 20 ml of distilled water and kept aside.

(b) A mixture of 20 ml phenol, 20 ml of lactic acid and 40 ml of glycerol was prepared by melting them together at 70°C.

The two (a) and (b) were then mixed and stored in dark colour bottle. It stains for nucleolus, RNA mitochondria cytoplasm and vacuoles.

(K) Red blue stain

Nucleic acids take double stain by Basic-Fuchsin-Methylene blue, DNA takes red whereas RNA takes contrasting blue colour. This stain is called as red blue double stain.

(L) Benzidine

This staining solution was prepared by dissolving 0.5 g Benzidine in 4.5 ml of Acetic acid bringing it to 100 ml with demineralized double distilled water. Prior to use 0.1 ml of 30 per cent H₂O₂ was added. It is used as a substrated for peroxidase enzymes in electrophoresis.

Enzymes

Separate stain used for enzymes-

(M) It is prepared by taking 6 ml sodium a-glycerophosphate in .01m acetate buffer and mixed it in 2ml of lead nitrate (.005m) and 1 ml of magensium chloride (0.05M). It gives test for Acid phosphatase by rendering brown black precipitation.

(N) Two types of stains were used for this test.

(a) Three per cent H₂O₂ of 5ml,0.1M phosphate buffer (pH 7) of 10
ml, 0.06M Na₂S₂O₃·5H₂O of 7ml of were mixed in 78 ml of water.

(b) 50 ml of 0.09M KI were prepared and added to 5ml of distilled water.

(O) Dissolved 7.1ml of catechal and 7.1ml of L-proline in phosphate buffer to make up to 0.1M(85.8 ml) solution. The bluish-orange bands appear.

GLYCERINE JELLY AS MOUNTING MEDIUM:

Soaked 7.0 g of granulated gelatin in 40 ml of distilled water for 30 minutes. It was then melted in a warm water bath and filtered through several layers of cheese cloth previously moistened with hot water.

A solution of phenol was prepared by taking 1.0 g of phenol (crystals) and mixing it to 50 ml of glycerine. This was finally added to the prepared gelatin, until a transparent mixture is formed.

HISTOCHEMICAL STUDIES

RESERVED FOOD MATERIALS

For the fixation of proteins, polysaccharides and lipids the reproductive organs of potato plants were fixed in FAA and Carnoy's fluid (Johanson, 1940) at room temperature for 24 hours. They were then preserved in 70 per cent ethanol. The dehydration was done in ethanol-xylol series. Thereafter the materials were embedded in Merck's paraffin wax.

The sections were cut at 10 to 12μ using a rotary microtome. The sections were dewaxed with xylene and dehydrated in downward series of ethanol.

The stain coomassie brilliant blue (R-250) was used for total proteins. The slides were kept in the stain for three minutes at 30°C. The excess stain was removed by 7 per cent acetic acid and the sections were mounted in glycerine jelly.

For polysaccharides the stain used were periodic
acid and Schiff's reagent (PAS). After rinsing with 70 per cent ethanol the sections were immersed in periodic acid solution for 5 minutes and then rinsed in 70 per cent ethanol. They were then kept in Schiff's reagent for 20 minutes at room temperature. After washing the sections with running water, they were mounted in Glycerine jelly.

The exclusive distribution of starch grains were localized by using IKI solution, using fresh hand sections as well as paraffin embedded sections of the tissues from the reproductive organs.

For staining lipids the sections were brought to 70 per cent ethanol and stained for 30 minutes in saturated Sudan black B prepared in 70 per cent ethanol at room temperature. The excess stain was immediately removed by rinsing the stained slides in 70 per cent ethanol. The sections were then washed in running water and counter stained in 1.0 per cent aqueous neutral red for one minute. The excess stain was removed by dipping in water before mounting in Glycerine jelly.

Nucleic acids were localized by using Basic Fuchsin -Methylene blue (red blue stain) which stain RNA blue and DNA red. Methylene blue stains RNA after keeping the sections for 30 to 50 minutes at room temperature whereas DNA is stained with Basic fuchsin by keeping sections for 30 minutes. The sections were washed with 70 per cent ethanol and mounted in Glycerin Jelly.

For the fixation of the enzymes acid phosphatase and peroxidase the reproduction potato plant organs were fixed in cold 100 per cent acetone at 4°C for 24 hours with two or three changes. The materials were dyhydrated with absolute ethanol and acetone followed by two change in benzidine for 45 minutes each. The materials were then embedded in paraffin at 56-58°C in the oven. The sections were kept
for incubation in a refrigerator till required. Sometimes fresh materials had also been used.

The sections were rinsed in 70 per cent ethanol or acetone for the enzyme acid phosphatase. They were then stained with the mixture of Sodium β-glycerophosphate: Lead nitrate: Magnesium chloride in the ratio 6:2:1. The slides with sections were kept for 45 minutes in incubator at 37°C. The sections were rinsed with water six times. The sections were further incubated in 1/50 (NH₄)₂ S for 2 minutes, which were rinsed with water and mounted in Glycerine jelly.

The enzyme peroxidase was localized by using the medium of saturated solution of benzidine; hydrogen peroxide (1 per cent) and Ammonium chloride (5%) in the ratio 5:5:1. The sections were first put in phosphate buffer at pH 7.0 and then transferred to the incubation medium (the above medium) for 2 minutes at room temperature. They were thoroughly washed and mounted in Glycerine jelly.

**ELECTROPHORESIS**

This apparatus was used for separation of Total Protein, Glycoprotein, Lipoprotein and Nucleic acids and Enzymes, in present study.

For such electrophoresis apparatus number of solutions are required of which most of them were prepared as stock and few as fresh ones.

1. **Tris glycine buffer**

(A) Tris, 6 g; glycine, 28.8 g made up to 1 litre with distilled water at a pH 8.3.

(B) Tris, 36.6 g in 1 mol/litre HCl, 48 ml; Tetramethyl - ethylene diamine (TEMED), 0.23 ml in distilled water made up to 100 ml. This results in a buffer at pH 6.7.

(C) Tris 6 g; 1 mol/litre HCl 48ml; TEMED, 0.46 ml in distilled water made to 100 ml with pH 6.7.
2. Acrylamide, 28 g; NN- methylene-bis-acrylamide GR (bis), 0.74 g; distilled water to make it to 100 ml.
3. Acrylamide, 10 g; GR (bis), 2.5 g; distilled water to make it to 100 ml.
4. Riboflavin, 4 mg; distilled water to make it to 100 ml.
5. Sucrose, 40 per cent in distilled water (100 ml solution).
6. Ammonium persulphate (a catalytic solution) 0.14 g make it to 100 ml in water.
7. Stains: - For staining different substances:
   (A) Total proteins - (a) Bromophenol blue
       (b) Coomassie brilliant blue R- 250
   (B) Lipoproteins - Sudan black B
   (C) Glycoproteins - Schiff's reagent
   (D) Nucleic acids - Methylene blue
   (E) Peroxidase - Benzidine.

**Preparation of samples**

The plant materials or samples used were of underground tuber; aerial tuber; sepals; petals; ovary; style; stigma; anther; pollen grains; fruits and seeds. An extract of each sample was prepared using the homogenizer.

To the homogenized material 40 per cent of freshly prepared sucrose was added. The whole mixture was then filtered. This renders a slightly viscous material (sample).

The above processing was done at low temperature.

Particular dyes were used by adding at a concentration of one drop.

To build up gel layers electrophoretic gel tubes were used. Two kinds of gel were prepared for two different layers.

**Separation gel**

This requires 1 part of B solution; 2 parts of no. 2
solution and 1 part of distilled water mixed with equal volume of the catalytic solution of Ammonium persulphate (no.6). Taking 1.0 ml of above mixtures, it was poured to each glass tube of the electrophoretic apparatus. An overlayer of one drop of distilled water was then carefully added. This gel was left for 20 to 40 minutes to set.

**Spacer gel**

To built up this layer 1 part of solution C; 2 parts of solution 3; 1 part of solution 4 and 4 parts of solution 5 were added one by one in a beaker and mixed well. This mixture is at pH 6.7 and termed as Spacer gel. The Spacer gel (0.2 ml) was then poured into the glass tubes above the separation gel layer (the Spacer gel was made fresh just before use).

One drop of distilled water was again added carefully layering the top of the "Spacer gel". These gel tubes were then exposed to fluorescent light for 40 minutes.

The gel layers were finally covered with a layer of dilute Tris-glycine buffer at pH 8.3 (1/10th dilution of the stock solution 1A). After polymerization the gel tubes were stored at 4°C for further use.

These gel tubes were fixed vertically in the disc with 12 holes as described by Davis (1964). The upper and lower chambers were then filled with 1/10th dilution of Tris glycine-buffer with pH 8.3.

The sample solutions of 0.1 ml amount was poured in each gel tube and one drop of stain was added.

The electrophoresis apparatus was kept at 4°C and connected to electrophoresis power supply. A strength of current 1.5mA/gel tube is passed through the gel tube for about 10 minutes. After 10 minutes the current is raised to 3 mA/gel tube. When the dye reached to the bottom of the gel tube the actual current kept for set of 12 gel tubes was
18 mA used. After this initial current strength the current was raised up to 30 to 35 mA. The current supply was stopped after 4 to 5 hours. The gels were then immersed in particular staining solutions and the excess stain was destained in 7 per cent acetic acid. This also helps in formation of destained bands which show more clarity and distinct separation (Loeschke and Stegemann, 1966; Desborough and Peloquin, 1966; 1967; 1968; Hames and Rickwood, 1981; Andrews, 1986; Plummer, 1988).