Chapter 1

General introduction and materials and methods
INTRODUCTION:

To meet the growing demand for plant products, there will be an increasing need for large numbers of plants of improved quality and shortened rotation. Perhaps the most effective way of increasing productivity is the use of genetically improved material as planting stock. Unfortunately this material is in short supply and therefore methods of repetitive propagation have been developed. One of the most extensive uses of tissue culture is the rapid clonal propagation of plants (Murashige, 1974, Vasil and Vasil, 1986).

The micropropagation is a non-conventional technique that allows plants to vegetatively multiplicate in shorter time and little spaces. In this method the plants are cultured in an aseptic environment, furnishing the optimal growth conditions by the use of culture media containing salts, vitamins, sugars, plant growth regulators and other organic compounds, under defined temperature, humidity, light intensity and light quality. In this way it is possible to rapidly stimulate the proliferation of new plants from a single bud or other parts of the plant, ensuring the health of the plant material and the preservation of the genetic uniformity. Since each bud or other parts of the plant can produce number of shoots, within a short period an exponential increase in the plant number can be expected.

Today, plant regeneration after harvest is often left to natural processes, although prompt artificial regeneration with selected genotypes provides the most effective means to increase the plant yield. At present, most of the planting material for diverse afforestation programs comes from sources that are genetically diverse and that give poor yield. Traditionally, practices by
plant breeders involving selection of superior plants in stands, propagating these to establish seed orchards, followed by breeding and progeny testing led to the production of improved seeds for breeding. However, most plants are difficult to propagate vegetatively and some method of clonal propagation is necessary to continue current plant breeding programs. Presently, the improved practices offer only limited possibilities to achieve that goal, and new technologies that can be used in addition to the older ones are required. Therefore, in order to enhance productivity, it is essential to have a highly efficient production technology that uses high yielding certified plant material. In this context, tissue culture and biotechnological tools can play an important role in boosting productivity (Bonga 1974; Bonga and Durzan 1987; Gupta et al., 1993; Reinert and Bajaj, 1995).

Tissue culture is the general term used to describe all the techniques in which cells or tissues are grown on special nutrient media under controlled environment. Micropropagation, which is a tissue culture technique, is a quick method of vegetative propagation.

The methods for vegetative propagation of plants can be divided into two categories:

(i) macropropagation through rooted cuttings, grafting and air layering; and

(ii) micropropagation through tissue culture, axillary branching, and induction of adventitious buds on cultured embryos or excised embryo parts, shoot tips, needle fascicles, unorganized callus or cell suspension cultures.
Despite high cost of micropropagation, a major advantage is the rapid multiplication of selected genotypes within a limited space. Although tissue culture methods are expensive, interest in this field has increased markedly over the past decade. The potential benefits of these techniques are now recognized by plant breeders.

The development of somatic embryos in vitro was first demonstrated by Steward et al., (1958). Some general principles for the induction of somatic embryogenesis have emerged. Usually, an auxin is required to induce somatic embryogenesis, and subsequent auxin withdrawal or lowering of the auxin concentration is necessary for embryo maturation (Dudits et al., 1991). In addition to the hormone regime, key variables are explant type and developmental stage, nutrition regime, and genotype (Ammirato, 1983; Rose et al., 1999). However, the molecular mechanisms involved in the induction of somatic embryogenesis are not well understood. There are many reports of genes involved in the regulation of the later stages of somatic embryogenesis (Zimmerman, 1993; Schmidt et al., 1997), but much less is known of the induction process. Ectopic expression of a number of different genes in Arabidopsis has been shown to induce vegetative cells to become embryogenic (Nolan et al., 2003).

Clonal propagation of superior plants of good form and resistance to diseases is of utmost importance. Somatic embryogenesis offers an alternative and efficient means for plant multiplication. In recent years, somatic embryos are being used for developing synthetic seeds, shortening the breeding cycle
and transformation studies. Somatic embryogenesis has been achieved in a number of dicotyledon and monocotyledon species

Regeneration of plants in vitro via somatic embryogenesis has some distinct features such as single cell origin and the consequent low frequency of chimeras and the production of a high number of regenerates (Ammirato, 1983). Somatic embryogenesis has several other advantages, including the efficiency of process (i.e. the formation of plantlets in fewer steps, with a concomitant reduction in labour, time and cost) and the morphological and cytological uniformity of the regenerants (Vasil and Vasil, 1986). Somatic embryogenesis has been achieved in many varieties of Mussaenda (Cramer and Bridgen, 1997), but to date there is no report on somatic embryogenesis in Mussaenda variety of 'scarlet' and 'rosea'. Plants regenerated via somatic embryogenesis may develop from a single cell, which reduces somaclonal variation. This advantage makes plant propagation more efficient (Mariani et al., 2002).

Direct somatic embryogenesis can also be used for genetic transformation (locco et al., 2001, Scorza et al., 1995, Franks et al., 1998). Transgenic plants can be generated by formation of embryogenic calli obtained from different tissues, including zygotic embryos (locco et al., 2001), leaves (Scorza et al., 1996), ovaries and anther filaments (Motoike et al., 2001).

Several woody plants often show poor rooting efficiency both in conventional (layering, cutting) and in vitro propagation. In the latter case it was possible to improve the in vitro rooting with hormonal application, dark
treatment, use of organic compounds, etc. (Damiano et al., 1991, Rugini et al., 1991, Ma et al., 1998), however, the difficulty of rooting is still one of the obstacle to successful micropropagation. Nowadays biotechnology is providing new soft methodology to overcome the use of synthetic auxins in inducing rooting.

Considerable progress had been made on the question of tissue nutrition. It soon came to be realized that no one single medium was satisfactory for the growth of all tissues, and this led to the formulation of different media to suit different tissues. Thus an impressive array of basic nutrient media came to be formulated within a few years, the most notable amongst them being that of White (1943), Heller (1953) and Nitsch (1951). The various formulations differed only in narrow limits, mostly in the concentration of individual ingredients e.g. macro and microelements or of omission of some and inclusion of others. A balanced solution, which served as a basic medium for a wide spectrum of plant tissues, is that of Murashige and Skoog (MS, 1962), Gamborg (B5, 1968) etc. and their several modifications.

The identification of cultivars and germplasm accessions through isozyme analysis offers for more precision than using morphological descriptions alone. Compared to other plant characters isozymes are quite rapidly screened, and relatively free of natural variation and simply inherited (Ryan and Scowcroft 1987). Isozymes is being used as a molecular marker showing modifications at the DNA level as expressed in their gene products (Brown et al., 1978, Hussain et al., 1987, Khephart 1990). Isozyme analysis facilitates establishment of genetic purity, location of duplicate accessions and
Identification and characterization of cultivars by isozymes of Esterase, Acid phosphatase and Peroxidase have been reported in barley and wheat (Bergman and Mann, 1973; Bassiri, 1976).

The present work was undertaken to study the micropropagation process of three varieties of Mussaenda viz. M. erythrophylla var. 'scarlet', M. erythrophylla var. 'rosea' and M. phillipica var. 'aurorae' and the medicinal plants, Wedelia chinensis, Eupatorium ayapana and Taxus wallichiana with the aim to optimize in vitro conditions for multiplication of these potential ornamentals as well as medicinal plants. The study was extended to protein and isozyme patterns to compare both donor plants as well as tissue culture raised plants of three varieties of Mussaenda, Wedelia and Eupatorium and study at the genomic level include karyotype analysis.

Aims of work:

1) Multiplication through shoot bud regeneration: The present work was undertaken to study the micropropagation process of three varieties of Mussaenda (M. erythrophylla var. 'scarlet', M. erythrophylla var. 'rosea' and M. phillipica var. 'aurorae'), and the medicinal plants, Wedelia chinensis, Eupatorium ayapana and Taxus wallichiana.

2) Multiplication through somatic embryogenesis: The study was extended to in vitro regeneration of plantlets through somatic embryogenesis from leaf derived callus culture of three varieties of Mussaenda.
3) **Regeneration via callus culture:** Both caulogenesis and rhizogenesis following callus development was achieved in *Wedelia, Eupatorium* and *Taxus*.

4) **Protein and isozyme study:** The protein and isozyme patterns were studied to compare both donor plants as well as tissue culture raised plants of three varieties of *Mussaenda, Wedelia* and *Eupatorium*. The study was extended to isozyme patterns to compare any variations among these three varieties of *Mussaenda*.

5) **Cytological aspects:** Studies at the genomic level include karyotype analysis. It also helps detection of true-to-type clone of donor plants as well as regenerated plants.
Materials and methods:

A. Tissue culture:

1) Explant source:

The young shoot buds (both terminal and axillary) and young leaf were used as explants. Shoot cuttings with the youngest two to five leaves and young leaf were collected from plants grown in the experimental garden of the institute. Material is collected fresh in the morning hours while the plants are still turgid, and not during midday when they tend to be flaccid. Material collected from the garden is invariably heavily contaminated with dust and microorganisms. After excision, the shoot tips (about one to three cms. in length) and leaf (about one to five cms. in length) were subjected to preliminary washing under running tap water for 10 minutes to 30 minutes which reduce the microflora to a substantial extent.

2) Media preparation:

Various types of culture media like Murashige and Skoog’s (1962), Gamborg’s B5 (1968) were used in the present study. Minor modifications to the above specifications when adopted are mentioned in the respective chapters. The growth hormones like auxins, gibberellins and cytokinins were added to the culture medium either alone or in various combinations according to the requirements and phases of growth.

3) Sterilization procedure:

In order to sterilize nutrient media, glass goods and instruments were autoclaved under steam at a pressure of 15 lb/in² and a temperature of 120°C for 15 minutes. However excised plant parts were surface sterilized to
remove the surface borne microorganisms. This procedure is done in front of a laminar airflow cabinet before the plant material is inoculated onto the culture medium.

The procedure adopted, the nature of the reagents used and time taken for sterilization is mentioned in detail at the beginning of the respective chapters.

B) Biochemical studies:

Extraction of total protein:

Reagents Used:

1. RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% Sodium deoxycholate and 0.1% SDS. The pH of the RIPA buffer was 7.5.

2. Laemmli's SDS-PAGE sample buffer (SSB) containing 0.76% Tris, 2% SDS, 10% Glycine, 0.005% Bromophenol blue. The pH was adjusted to 6.8. Then 10% β-marcaptoethanol was added to the buffer.

4) Extraction Procedure:

Leaves of three varieties of *Mussaenda* (both explants as well as tissue culture raised plants) were excised and kept at -70°C. Then the leaves were crushed in an ice-cold mortar pestle with ice-cold RIPA buffer. The homogenate was collected and centrifuged in a RC5B Sorvall Refrigerated Centrifuge for one hour at 12,000 rpm. The clear supernatant was collected and again centrifuged in a Beckman L7-55 Ultracentrifuge at 41,000 rpm for two hours.
After centrifugation the pellet was discarded and the supernatant was collected and kept in -20°C and used up within a fortnight.

Estimation procedure:

(I) By Lowry's method (1951):

This is the most common useful method for estimation of proteins. However presence of Bromophenol, β-marcaptoethanol in samples, interferes in the estimation.

Reagent:

Lowry A – 2% Sodium carbonate was dissolved in 0.1 (N) NaOH.

Lowry B1 – 1% Sodium Potassium Tartarate was dissolved in distilled water.

Lowry B2 – 0.5% Copper sulphate was dissolved in distilled water.

Reagent C – This was prepared just before use. Lowery A, B1 and B2 were mixed in 98:1:1 ratio.

Folin-Ciocalteau Reagent – 1 part of Folin was dissolved with 2 parts of water.

Assay procedure:

1. In all the test tubes 4 ml of Reagent C was taken.
2. Desired amount of protein samples (eg. 10μl, 20μl, .......) was added to each tube excepting the blank.
3. In the blank equal amount of RIP A buffer was added.
4. The tubes were vortexed and incubated 10 minutes at room temperature.
5. Thereafter 0.5 ml of Folin reagent was added to all tubes, vortexed and incubated at room temperature for 20 minutes. A blue colour developed in the tubes containing protein solution.
6. The intensity of the colour was measured in UV Spectrophotometer at 680 nm.

**Polyacrylamide-Sodium dodecyl Sulphate Gel Electrophoresis (SDS-PAGE) of proteins:**

Almost all analytical electrophoresis of proteins was carried out in polyacrylamide gels under conditions that ensured dissociation of the protein into their individual polypeptide sub-units and that minimized aggregation. Most commonly, the strongly anionic detergent SDS was used in combination with a reducing agent and heat to dissociate the complex proteins before they were loaded on the gel. The denatured polypeptides bind SDS, and the amount of SDS bound was almost proportional to the molecular weight of the polypeptide and was independent of its sequence. So the SDS-polypeptide complexes migrated through polyacrylamide gels in accordance with the size of the polypeptides. In most cases, SDS-PAGE was carried out with a discontinuous buffer system originally devised by Ornstein and Davies (1964). Use of this discontinuous buffer system increased the desolution of SDS-PAGE by concentrating all of the complexes in the sample in a very small volume.

**Preparation of SDS-Polyacrylamide Gel:**

**Reagents:** Acrylamide and N,N'-methylenbisacylamide.

A solution of acrylamide-bisacylamide (Sigma) was prepared in 30:0.8 ratio (dissolved in deionized, warm water). Acrylamide and bisacylamide were slowly converted during storage to acrylic acid and bis-acrylic acid. This deamination reaction was catalyzed by light and alkali. So, pH of the solution was kept at 7 and stored in dark bottles.
Sodium dodecyl sulfate (SDS, Sigma): A 10%(w/v) stock solution was prepared in deionized water and stored in room temperature.

**Tris buffer (for the preparation of resolving and stacking gels):**

**Resolving Gel:**

For the resolving gel buffer 9% Tris (Sigma) was dissolved in de-ionized water and the pH was adjusted to 8.8 with concentrated HCl. 0.2% SDS was added to make the Tris HCl buffer for resolving gel.

**Stacking gel:**

For the stacking gel buffer 3.08% Tris (Sigma) was dissolved in de-ionized water and the pH was adjusted to 6.8 with concentrated HCl. To this 0.2% SDS was added.

TEMED (N, N, N',N'-tetrabutylethylenediamine): TEMED accelerates the polymerization of acrylamide-bisacrylamide (Sigma) by catalyzing the formation of free radicals from ammonium persulfate.

**Ammonium persulfate:**

Ammonium persulfate provides the free radicals that drive polymerization of acryl-amide and bisacrylamide. A 10% (w/v) stock solution was prepared by dissolving 100 mg of APS in 1 ml of de-ionized water and stored at 4°C.
Tris-glycine electrophoresis buffer:

Tris buffer contains 3.03 gm of Tris (Sigma), 14 gm of Glycine (Sigma) and 1 gm of SDS (Sigma) dissolved in 1000 ml of de-ionized water and the pH was adjusted to 8.3.

Composition of Laemmli’s sample buffer (2 X SSB):

It contains 2% SDS, 100 mM DTT, 60 mM Tris and 0.001% bromophenol blue.

All the chemicals were dissolved in double distilled water and the pH was adjusted to 6.8. The DTT was added to 2 X SSB before loading.

1 M DTT stock:

First 0.01 M Sodium acetate was prepared and kept in -20°C as stock. 1 M DTT was prepared by dissolving it in 1 ml Sodium acetate (from stock) and kept as stock in -20°C. 100 mM DTT was obtained from the stock and mixed with 2 X SSB before loading the protein.

Stain:

0.1% Coomassie Brilliant Blue R-250 (Merck) was dissolved in Water: Methanol: Glacial acetic acid in 5:5:2 (w/v), filtered and stored.

Destainer:

100 ml of Methanol, 17.5 ml of Glacial acetic acid and 135 ml of distilled water were mixed together and stored at room temperature.

6H Molecular weight marker purchased from Genei (Bangalore). All the chemicals were dissolved in double distilled water.
*Apparatus:*

The use of discontinuous buffer system requires SDS-polyacrylamide electrophoresis to be carried out in vertical gels. The apparatus used in the present experiments was purchased from BIORAD (GERMANY).

*Pouring SDS-polyacrylamide gels:*

1. Glass plates were assembled.
2. In an Erlenmeyer flask, the appropriate volume of solution was prepared, containing 2.5 ml of acrylamide-bisacrylamide solution, 3.8 ml of resolving gel buffer (pH 8.8), 1.23 ml of distilled water, 50μl Ammonium persulfate (APS) and 5 μl of TEMED. Polymerization began as soon as the TEMED had been added. The mixture was swirled rapidly.
3. The solution was poured into the gap between the two plates with the help of a Pasteur pipette leaving sufficient space for the stacking gel. The gel was carefully overlayed with Isobutanol or distilled water.
4. After polymerization was complete, the overlay was poured off and top of the gel was washed with distilled water for several times to remove any unpolymerized acrylamide.
5. The stacking gel was prepared as follows: 1 ml of acrylamide-bisacrylamide, 3 ml of stacking gel buffer (pH 6.8), 2 ml of distilled water, 50 μl of Ammonium persulfate (APS) and 7 μl of TEMED mixed thoroughly.
6. Without any delay the stacking gel was poured onto the surface of the polymerized resolving gel. Immediately a Teflon Comb was inserted into the stacking gel solution in such a way that no air bubble was trapped.
7. While the stacking gel was polymerizing the sample proteins were prepared. Desired samples of proteins were mixed in the sample buffer (2 X SSB + DTT), boiled for 3 minutes and then centrifuged at 10,000 rpm for 2 minutes.

8. After polymerization was complete, the Teflon Comb was removed. Using a squirt bottle wells were washed and the gel was mounted in the apparatus. Running buffer was loaded to the reservoirs. Trapped air bubbles were removed.

9. Required amount of (100-150 µg) protein samples were loaded to each well with the help of a micropipette fitted with multiflex tips (MULTI, USA).

10. After the loading the apparatus was attached to an electric power supply. The gel was run at constant current of 60-80 voltage.

11. When the run was completed, the power supply was switched off and the glass plates having the gels were removed from the apparatus. The stacking gel part was cut off and the rest was stained with Coomassie Blue for overnight. The stained was discarded and the gel was de-stained. After proper de-staining it was photographed.

**Detection of Isozymes:**

**Extraction of total protein:**

**Materials:**

Leaves of three varieties of *Mussaenda* viz. 'scarlet', 'rosea' and 'aurorae' (both donor plants and regenerated plants).
Reagents:

Composition of PEB buffer:

0.1 M Tris-HCl, 0.25 M Sucrose, 1% Polyvinylpyreledon (PVP), 1% Ascorbic acid, 0.1% Cystein HCl, 1 mM EDTA, 0.4 mM MgCl₂, 0.4 mM DTT, 1% β-marcaptoethanol. All the chemicals were dissolved in double distilled water and the pH was adjusted to 6.8.

The chemicals were dissolved one by one. DTT and β-marcaptoethanol was added before electrophoresis. The pH was measured and adjusted by adding concentrated HCl and 1 (N) HCl and not NaOH. PEB was autoclaved and stored in -20°C.

Procedure:

*Mussaenda* leaves were excised and kept in -70°C for some days to avoid chlorophyll interference. The materials were crushed in an ice-cold mortar pastle with ice-cold PEB buffer in cold room. The homogenate was collected and centrifuged in a RC 5B Sorval Refrigerated centrifuge for 45 minutes at 12,000 rpm. The clear supernatant was collected and again centrifuged in a Beckman L7-55 Ultracentrifuge at 40,000 rpm for 2 hours. After centrifugation, the pellet was discarded and the supernatant was lyophilized for 10-12 hours as required.

Acetone precipitation:

Acetone precipitation was done, as PVP, which was present in PEB buffer, was likely to interfere in the estimation of Isozyme.
The supernatant obtained was divided in several eppendorf tubes. One eppendorf was used for acetone precipitation and rest eppendorfs were stored in -20°C for further use. The supernatant was used directly to do the work.

Twice the volume of chilled acetone was added to one eppendorf and incubated at -20°C for 30 minutes. The precipitate was collected by centrifugation in Microfuge RM 12C (Remi equipment) at 8,000 to 10,000 rpm for 5 minutes.

The acetone was discarded, fresh acetone was added, mixed well and the process was repeated for 3 to 4 times. The above mentioned step was carried out to purify the content. The eppendorf tube was then kept open for sometime so that the acetone was volatilised. The precipitate was then dried properly in Vacuum drier and stored in -20°C for further use.

Before use, one eppendorf containing precipitate sample was taken and then the sample was recovered by dissolving in 0.1 (N) NaOH. This was used for estimation of isozyme. To other eppendorfs, sample buffer without Bromophenol blue was added and used for further process.

**Estimation of proteins:**

**By Lowry's Method (1951)**

This is the most common and useful method for estimation. Presence of PVP in PEB buffer interferes in the estimation. So, before estimation, acetone precipitation has to be done.
Reagent:

Lowry A – 2% Sodium carbonate was dissolved in 0.1 (N) NaOH.

Lowry B1 – 1% Sodium Potassium Tartarate was dissolved in distilled water.

Lowry B2 – 0.5% Copper sulphate was dissolved in distilled water.

Reagent C – This was prepared just before use. Lowery A, B1 and B2 were mixed in 98:1:1 ratio.

Folin-Ciocalteau Reagent – 1 part of Folin was dissolved with 2 parts of water.

Assay procedure:

Acetone precipitation has to be done before estimation of Isozymes. The precipitated protein was recovered by dissolving it in 0.1 (N) NaOH solution. The volume of 0.1 (N) NaOH solution added being not more than the volume of the supernatant collected after cold centrifugation. The solution was vortexed intensely till the entire pellet dissolves.

1. In all the test tubes 4 ml of Reagent C was added.
2. Desired amount of protein samples (eg. 10μl, 20μl, ....) were added to each tube excepting the blank.
3. In the blank equal amount of 0.1(N) NaOH buffer was added.
4. The tubes were vortexed and incubated for 10 minutes at room temperature.
5. Thereafter 0.5 ml of Folin reagent was added to all the tubes, vortexed and incubated at room temperature for 20 minutes. A blue colour developed in the tubes containing protein solution.
6. The intensity of the colour was measured using UV Spectrophotometer at 680 nm.
**Polyacrylamide gel electrophoresis:**

Technically the sample extract is electrophoresed in polyacrylamide buffer (non denaturing with no SDS) at low temperature (4-8°C).

Polyacrylamide gels are composed of chain of polymerised acrylamide that are cross linked by a bifunctional agent such as N, N'-Methylene bisacrylamide. Cross-links formed from bisacrylamide added rigidity and tensile strength to the gel and from pores through which complexes pass.

**Preparation of SDS-Polyacrylamide Gel:**

**Reagents:** Acrylamide and N,N'-methylenebisacrylamide.

A solution of acrylamide-bisacrylamide (Sigma) was prepared in 30:0.8 ratio (dissolved in deionized, warm water). Acrylamide and bisacrylamide were slowly converted during storage to acrylic acid and bis-acrylic acid. This deamination reaction was catalyzed by light and alkali. So, pH of the solution was kept at 7 and stored in dark bottles.

**Tris buffer (for the preparation of resolving and stacking gels):**

**Resolving Gel:**

For the resolving gel buffer 9% Tris (Sigma) was dissolved in de-ionized water and the pH was adjusted to 8.8 with concentrated HCl.

**Stacking gel:**

For the stacking gel buffer 3.08% Tris (Sigma) was dissolved in de-ionized water and the pH was adjusted to 6.8 with concentrated HCl.
TEMED (N, N, N', N' - tetramethylethylenediamine): TEMED accelerates the polymerization of acrylamide-bisacrylamide (Sigma) by catalyzing the formation of free radicals from ammonium persulfate.

**Ammonium persulfate**: Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. A 10% (w/v) stock solution was prepared by dissolving 100 mg of APS in 1 ml of de-ionized water and stored at 4°C.

**Tris-glycine electrophoresis buffer**: Tris buffer contains 3.03 gm of Tris (Sigma), 14 gm of Glycine (Sigma) and no SDS dissolved in 1000 ml of deionized water and the pH was adjusted to 8.3.

**Composition of sample buffer**: This buffer was prepared by dissolving 0.76% Tris in double distilled water and 10% Glycerol was added to it. 0.001% Bromophenol blue was added before loading. The pH was adjusted to 6.8.

**Apparatus**: The polyacrylamide electrophoresis was carried out in vertical gels. The apparatus used in the present experiments was purchased from BIORAD (GERMANY).
Pouring SDS-polyacrylamide gels:

1. Glasses, plates were assembled.

2. In a beaker, the appropriate volume of solution was prepared by mixing thoroughly the following
   i) 2.5 ml of acrylamide-bisacrylamide solution,
   ii) 3.8 ml of resolving gel buffer (pH 8.8),
   iii) 1.23 ml of distilled water, iv) 50 μl Ammonium persulfate (APS) and v) 5 μl of TEMED. Polymerization began as soon as the TEMED had been added. The mixture was swirled rapidly.

3. The solution was poured into the gap between the two plates with the help of a Pasteur pipette leaving sufficient space for the stacking gel. The gel was carefully overlayed with Isobutanol or distilled water.

4. After polymerization was complete, the overlay was poured off and top of the gel was washed with distilled water for several times to remove any unpolymerized acrylamide.

5. The stacking gel was prepared as follows: 1 ml of acrylamide-bisacrylamide, 3 ml of stacking gel buffer (pH 6.8), 2 ml of distilled water, 50 μl of Ammonium persulfate (APS) and 7 μl of TEMED mixed thoroughly.

6. Without any delay the stacking gel was poured onto the surface of the polymerized resolving gel. Immediately a Teflon Comb was inserted into the stacking gel solution in such a way that no air bubble was trapped.

7. While the stacking gel was polymerizing the sample proteins were prepared. Equal volume of supernatant extract was mixed with sample buffer. To it 0.001% Bromophenol blue was added and vortexed and centrifuged for 2 seconds.
8. After polymerization was complete, the Teflon Comb was removed. Using a squirt bottle wells were washed and the gel was mounted in the apparatus. Running buffer was loaded to the reservoirs. Trapped air bubbles were removed.

9. Each lane was loaded with equal amount of proteins after normalizing the protein content in extract as small volume as possible. 150 µg of protein samples were loaded to each well with the help of a micropipette fitted with multiflex tips (MULTI, USA).

10. After the loading the apparatus was attached to an electric power supply. The gel was run at constant current 60-80 voltage. The entire operation was performed in an air-conditioned room. Ice bags and cubes were placed all around the apparatus to maintain low temperature.

11. When the run was completed i.e. as the tracking dye reached the anodic end, the power supply was switched off and the glass plates having the gels were removed from the apparatus. The stacking gel part was cut off and the rest was incubated in respective buffer and substrate solution of respective Isozymes.

**Acid phosphatase:**

Acid phosphatase Isozymes was visualized in the gel by the following methods as described by Wetter and Dyke (1983).

**Solution and regents:**

For preparation of 500 ml of 0.15 (M) Acetate buffer 2.5 gm of NaOH was dissolved in 100 ml of distilled water. 4.65 ml of Glacial acetic acid was
added to it. The pH was adjusted to 5.0 by adding acetic acid drop by drop. The final volume was adjusted by adding distilled water.

Staining solution (40 ml):

0.05 gm of 1-napthyl acid phosphate was dissolved in 20 ml of 0.15 (M) acetate buffer and then filtered on Whatman filter paper.

0.05 gm Fast blue RR was dissolved in 20 ml 0.15 (M) acetate buffer and filtered. Both the staining solutions were mixed.

After electrophoresis the gel was incubated for one hour in 0.15 (M) acetate buffer (at 4°C) and washed 3-4 times. The gel was incubated in the staining solution for overnight at 4°C and then washed with water. The gel was stored in 1% acetic acid.

Esterase:

Esterase Isozymes were visualized in the gel by the following methods as described by Wetter and Dyke (1983).

Solution and regents:

To prepare 500 ml of 0.1 (M) Sodium phosphate buffer 6.25 gm of NaH₂PO₄ and 1.40 gm of NaHPO₄ was dissolved separately in 100 ml of distilled water. Both the solutions were mixed. To adjust the pH at 6.0, acetic acid was added drop by drop and final volume was made up by adding distilled water.
Staining solution (40 ml):

0.02 gm of 1-napthyl acetate was dissolved in 200 µl of acetone and then 20 ml 0.1 (M) Sodium phosphate buffer was added to it. 0.04 gm of Fast Garnet GBC mixed and filtered on Whatman filter paper.

After electrophoresis the gel was incubated for ½ hour in 0.1 (M) Sodium phosphate buffer (at 4°C). The buffer was changed at every 15 minutes. The gel in the staining solution was incubated for 15-20 minutes at 4°C and then washed with water. The gel was stored in 1% acetic acid.

Peroxidase:

Guaicol- H_2O_2 method (Hislop and Stahmann, 1971) was followed for the visualization of peroxidase isozymes.

Solution and regents:

For preparation of 500 ml of 0.15 (M) Acetate buffer 2.5 gm of NaOH was dissolved in 100 ml of distilled water. Then 4.65 ml of Glacial acetic acid was added to it. The pH was adjusted at 5.0 by adding acetic acid drop by drop. The final volume was adjusted by adding distilled water.

Staining solution (40 ml):

To prepare 40 ml of staining solution, 20 ml of 0.15 (M) Acetate buffer, 50 µl of Guaicol and 50 µl of H_2O_2 were added gradually. After electrophoresis the gel was incubated for ½ hour in 0.1 (M) Sodium phosphate buffer (at 4°C). After every 15 minutes the buffer was changed. The gel was
incubated in the staining solution for 5-10 minutes at 4°C and then washed with water. Then the gel was stored in 1% acetic acid.

C) Cytological studies:

Materials:

Freshly excised root tips of three varieties of Mussaenda viz. ‘scarlet’, ‘rosea’ and 'aurorae' (both explants and tissue culture raised plants). Wedelia chinensis, Eupatorium ayapan.

Reagents:

i) Fixatives: Acetic acid Ethanol mixture (1:3): Glacial acetic acid and Ethanol were mixed in the ratio of 1:3 and stored in room temperature.

ii) 45% Acetic acid: 45 ml of Glacial acetic acid was mixed well with 55 ml of distilled water to make the volume 100 ml and stored at room temperature.

iii) 2% Aceto-Orcein stain: 2 gm of Orcein powder (E. Merck, India) was weighed and dissolved gradually in 100 ml of boiling 45% Acetic acid. The solution was heated for 10 minutes, carefully keeping it at a simmering point. When the solution cooled down to room temperature it was filtered and stored in a clear bottle. Before use 9 parts of Aceto-Orcein solution was mixed with 1 part 1(N) HCl.

iv) PDB: A saturated solution of Para-dichlorobenzene was prepared in distilled water and kept in a bottle for several months at room temperature.
Method:

Fresh root tips were pretreated in saturated Para-dichlorobenzene solution (PDB) at 10-12°C for 3 to 4 hours. Then the roots were washed and fixed in Acetic acid: Ethanol mixture (1:3) for overnight. Next day, root tips were placed in 2% Aceto-Orcein: HCl (9:1). After slight warming the root tips were kept in the stain for 1 hour. Later the roots were squashed in 45% acetic acid and sealed with wax. Micrographs were taken with the help of Photoautomat Wild-Leitz (Leico) Photomicroscope.