CHAPTER 2

Materials and Methods
INTRODUCTION

This chapter describes the materials, general techniques routinely practiced in plant tissue culture and methods used for enzyme activity measurement, lipid peroxidation estimation and metal estimation. The materials and methods, specific to particular experiment, are dealt in details in respective chapters.

A. Materials

Most of the consumables and chemicals were procured from various local suppliers.

Glasswares and Plasticwares:

Test tubes (25x150 mm), conical flasks (250 ml capacity), pipettes (0.1, 0.2, 1, 2, 5, 10 ml capacity) and measuring cylinders (25 ml, 100 ml, 1000 ml capacity) of Borosil, India were used for culturing the tissues and for preparation of media. Autoclavable, screw cap bottles (100, 250 & 500 ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (60 ml capacity), slides (Blue Star, India) and cover slips (Micro-Aid, India).

Plasticwares including sterile disposable plastic petriplates of 55 and 85 mm diameter were procured from Tarson, Pune. Klin wrap, used for sealing the petriplates. Micropipette of different precision measurements (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl and 2 µl) and microtips were procured from Gilson and Tarson respectively.

Chemicals:

Chemicals used for surface sterilization procedures were Bavistin® (BASF, India), Savlon (Johnson and Johnson Limited, USA) and Mercuric chloride (Qualigens Fine Chemicals, India).

Inorganic salts and vitamins used for preparation of culture media and for other experiments were of Analar grade (BDH, Hi-Media and Qualigens Fine Chemicals, India). Sucrose, Agar agar (bacteriological grade), used as gelling agent in the semisolid culture medium was procured from Hi-Media (India).
Chemical used for enzyme extraction and enzyme assay including sodium dihydrogen phosphate, disodium hydrogen phosphate, triton-x-100, polyvinylchloride were procured from Himedia. Hydrogen peroxide was procured from Qualigens Fine Chemicals, India and guaiacol, methionine, nitobluetetrazolium, EDTA, riboflavin were procured from Sigma (U.S.A.).

Chemicals including trichloroacetic acid and thiobarbituric acid were obtained from Sigma (U.S.A.) for lipid peroxidation estimation. Chemical used for metal estimation including perchloric acid and nitric acids were procured from Qualigen fine chemicals, India.

For histological studies formaldehyde solution, glacial acetic acid and xylene were procured from Qualigens Fine Chemicals, India. Ethanol, 2-methyl propan-2-ol (tert butyl alcohol), iron alum was from S.D. fine chemicals, India. Paraffin wax (m.p. 58-60°C) was from (E. Merck, India Ltd.); Haematoxylin and Eosin stain was from Hi-Media Laboratories Pvt. Ltd., Bombay, and DPX-4 mountant [189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)] was from BDH, India.

Equipments:
The major equipments used include,

\textbf{pH meter (Thermo Orion A+):}

pH is the negative logarithm of hydrogen ion concentration. The measurement of pH in pH meter (Thermo Orion) is based on ion exchange in between hydrated layers formed on glass surface. Change in ion exchange results in emf or voltage difference causing current flow. The current intensity gives the value of pH. The pH meter consists of two electrodes a calomel electrode and a glass electrode. The glass electrode contains a silver chloride and 0.1 N hydrochloric acid. Its tip is covered by a special glass surface, which allows only H\(^+\) ions to pass through it. The zero of the dial is first set by mechanical means. Then the knob for temperature compensation is adjusted for the temperature of the solution. This is essential since equilibrium constant of a reaction does vary with temperature. As a next step, the electrodes are dipped in to a standard buffer solution (This is the solution whose \([H^+]\) is accurately known) of known pH. The dial read this value. The electrodes are now removed,
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washed well with distilled water, and dipped into the unknown solution. The dial reads the pH value of the solution.

**Electronic Balance (Contech):**

A manual top loading balance was used for quick weighing and for analytical purposes. This is a single pan balance of capacity 100-200 gm, sensitivity 0.1 mg operating on 230 V 50 Hz AC mains. Precision of ± 0.005 g, weighing range 0-1, 200 g, digital read out was used for making stock solutions of growth regulators and for other fine weighing.

**Autoclave:**

The autoclave (Nat Steel Equipment Private Limited, Mumbai, India) was used for sterilization of media, glasswares, water, dissecting instruments, etc. and also for decontamination of contaminated cultures. It is based on the application of steam under pressure. High pressure provides high boiling point than normal temperature that contains more heat content, which causes coagulation of proteins and cell death. It has a thermostat with a double-jacketed steam chamber. Thus the temperature and pressure can be maintained. The culture media are autoclaved at 121°C and 15 lb/inch² pressure for 20 minutes. Glass wares and other materials are autoclaved for 1h.

**Laminar airflow ultra clean unit (Micro-Filt, Pune, India):**

It is a bench on which all aseptic manipulations are carried out. In laminar with the help of an air pump, air is passed through the HEPA filters. The pore size of the filter is 0.22 microns. The fan fitted in this unit pushes the air through the filter at high pressure from one side creating positive pressure inside the chamber. So the entry of any contaminant is prohibited from the open side of the bench. The instrument is fitted with UV tubes in addition to the fluorescent tubes. Before using the instrument UV is put on for 15 to 20 minutes and bench is to be cleaned with 96% alcohol swab to eliminate microorganisms. The laminar used for present studies was a horizontal laminar type. Here airflow is in horizontal direction. It is the most common type used in tissue culture labs.
Atomic Absorption Spectrophotometer 1100B (Perkin Elmer, USA):

Atomic absorption spectroscopy (AAS) determines the presence of metals in liquid samples. In their elemental form, metals absorb ultraviolet light when excited by heat. Each metal has a characteristic wavelength that will be absorbed. The AAS instrument looks for a particular metal by focusing a beam of UV light at a specific wavelength through a flame and into a detector. The instrument measures the change in intensity. The determination of element is based on atomic absorption. When light emitted by the lamp passes from monochromator, it becomes plane-polarized light. The intensity of flame and lamplight produces a resultant intensity. When element absorbs light there is a decrease in intensity and this is detected as a flash by photomultiplier. Flash and light intensity decrease is directly proportional to concentration of the element. A computer data system converts the change in intensity into an absorbance. The sensitivity of the instrument used in this study is 2 ppm. Initially standard sample is passed to determine the original wave absorbed. Then the sample is passed and concentration is determined.

Centrifuge:

Centrifuge is an instrument which is based upon the principle of centrifugation separation process which works on an applied centrifugal field, such as the relative molecular mass, shape and density. The basis of centrifugal techniques is to exert a larger force than does the earth’s gravitational field, thus increasing the rate at which the particles sediment. Particles that differ in density, shape or size can be separated because they sediment at different rates in centrifugal field, each particle sedimenting at a rate that is directly proportional to the applied centrifugal field. Centrifuge was used for isolation of tissue extract used for enzymes activity estimation and protein estimation.

Other instruments used in the course of the present study include Magnetic stirrer (Remi, India), Steamer (Ultradent, India), Temperature controlled oven (Pathak Electricals, India), Light microscope (Carl-Zeiss Jena), Microtome (Reichert Jung), Camera (Nikon/Zeiss), membrane filter sterilizing unit (Laxbro, Pune) and Pipetman (Gilson/Tarson) were used. With the exception of pipetman, microtome, microscopes
and camera, all other equipments used in the course of this study are fabricated by
different companies in India.

B. Methods

Preparation of glassware and instruments:

Glassware used in our studies was cleaned by boiling in saturated solution of
sodium bicarbonate for 1 h followed by washing in tap water. These were then
immersed in 30% nitric acid solution for 30 min and were washed thoroughly with
tap water. After rinsing with double distilled water these were allowed to dry on a
draining rack.

Tubes and flasks were plugged with absorbent cotton (Safe Surgical
Industries, Beawar, India). All dissecting instruments were either wrapped singly or
were put in closed aluminum cans for sterilization by autoclaving. Ordinary grade
filter paper pieces of approximately 10x20 cm were kept in stack alternatively with
brown paper pieces of similar size. These were packed in autoclavable plastic bags
with 20-25 pieces in each bag and autoclaved. Dissection and transfer of explants
were carried out on these papers under aseptic conditions and disposed after use.
Pipet tips used for aseptic addition by micropipets were arranged in cases meant for
their size, wrapped with brown paper and autoclaved. Sterilization of the glassware
and instruments was carried out by autoclaving at 121°C for 1 h in 15 lbs/(inch)^2.

Preparation of media:

Success of a tissue culture protocol depends on the appropriate composition of
the medium. Several basal formulations like Murashige’s and skoog media, Schenk
and Hildebrandt media. Concentrations of the macro and microelements, salts and
organic constituents of the MS (Murashige and Skoog, 1962), modified SH (Schenk
and Hildebrandt, 1972) basal medium are listed below. Stock solutions of the media
ingredients were prepared by dissolving weighed amounts of these salts as given
below in distilled water. Appropriate aliquots of these solutions were mixed to
prepare the media.
COMPOSITION OF MURASHIGE AND SKOOG'S BASAL MEDIUM

(Composition of the stock solutions)

**MS MAJOR (1000 ml) 20X**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>KNO₃</td>
<td>38.16 grams</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>33.16 grams</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>8.96 grams</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>7.56 grams</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.56 grams</td>
</tr>
</tbody>
</table>

**MS MINOR (500 ml) 100X**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
<td>310 mg</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>1115 mg</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>430 mg</td>
</tr>
<tr>
<td>Na₂MoO₄</td>
<td>12.5 mg</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>1.25 mg</td>
</tr>
<tr>
<td>KI</td>
<td>41.5 mg</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>1.25 mg</td>
</tr>
</tbody>
</table>

**VITAMINS (500 ml) 100X**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine amide</td>
<td>25 mg</td>
</tr>
<tr>
<td>Glycine</td>
<td>100 mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>5 mg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>25 mg</td>
</tr>
</tbody>
</table>

**INOSITOL 100X**

5 grams of Inositol was dissolved in 500 ml of distilled water.

**Fe-EDTA (500 ml) 100X**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄</td>
<td>1.390 grams</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.865 grams</td>
</tr>
</tbody>
</table>
COMPOSITION OF SCHENK AND HILDEBRANDT (SH) MEDIA

(Composition of the stock solutions)

**SH MAJOR (500 ml) 20X**
- KNO₃ 25 grams
- CaCl₂·2H₂O 2.0 grams
- MgSO₄·7H₂O 4.0 grams
- NH₄H₂PO₄ 3.0 grams

**SH MINOR (100 ml) 100X**
- H₃BO₃ 50 mg
- MnSO₄ 100 mg
- ZnSO₄ 10 mg
- Na₂MoO₄ 1 mg
- CoCl₂·6H₂O 1 mg
- KI 10 mg
- CuSO₄·5H₂O 2 mg

**SH VITAMINS (100 ml) 100X**
- Nicotine amide 5 mg
- Thiamine 50 mg
- Pyridoxine 5 mg

**INOSITOL 100X**
10 g of Inositol was dissolved in 100 ml of distilled water.

**Fe-EDTA (100 ml) 100X**
- FeSO₄ 150 mg
- Na₂-EDTA 200 mg
Stock solutions of growth regulators (GR) were prepared by adding few drops of solvent in the required amount of growth regulator to dissolve. After dissolution, the required concentration was made by the addition of double distilled water and stored in refrigerator in sterilized bottles.

For media preparation a calculated amount of aliquots were added from these stock solutions. Unless mentioned, pH of all the media was adjusted to 5.6-5.8 using 1N NaOH or 1N HCl after mixing all the constituents except the gelling agent. The volume was made up with double distilled water. Gelling agent (agar agar) was then added and heated on water bath or steamed for the agar to melt. Molten medium was dispersed into sterile culture tubes (20 ml of media), flasks (100 ml of media) or bottles (80 ml of media) after thorough mixing. Semisolid medium containing agar was used in most of the studies unless otherwise mentioned. All the culture media were autoclaved for 20 min. at 121°C and 15 lbs/(inch)^2.

**Culture conditions:**

Cultures were incubated in light in culture room adjusted at 25±2°C with 16 h photoperiod at 32 μE m^-2 s^-1 light intensity.

**Histological Techniques:**

Sections were prepared for histological studies following the methods described by Sharma and Sharma, 1980. The tissues were cut into small pieces (approx 3 x 4 mm) and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48 h at room temperature. Tissues were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (mp 58-60°C). Serial sections of 10 μM were cut using a rotary microtome (Reichert-Jung 2050, Germany). Sections were double stained with haematoxylin-eosin and mounted with DPX (Loba Chemie, Mumbai, India) for studies under microscope.

**Filter sterilization:**

The solutions and liquid used in the experiments was filter sterilized by passing through membrane filters. All the particles, microorganisms and viruses which are bigger than the pore diameter of the filter used (0.22 μm) are removed. The
greatest advantage of this method is that thermolabile substances like vitamins, amino acids, hormones viz., GA3, zeatin, Abscisic acid (plant growth regulators) can be sterilized unchanged. In present investigation K$_2$Cr$_2$O$_7$, CuSO$_4$ and CdCl$_2$ solution were filter sterilized.

**Lipid Peroxidation:**

The reaction of lipid peroxides with thiobarbituric acid (TBA) has been widely adopted as a sensitive assay method for lipid peroxidation in animal tissues (Ohkawa et al., 1979). In the present investigation, the level of lipid peroxidation products and thiobarbituric reacting substances (TBARS) in different tissues was estimated following the modified method used in studies on arsenic tolerance in ferns (Srivastava et al., 2005). Approximately 0.250 g of frozen plant tissue samples was cut into small pieces, homogenized with the addition of 2.5 ml of 5% trichloroacetic acid, and centrifuged at 10000 g for 15 min at room temperature. Equal volumes of supernatant and 0.5% thiobarbituric acid in 20% trichloroacetic acid were added in a new tube and incubated at 96 °C for 25 min. The tubes were transferred into an ice bath and then centrifuged at 8000 g for 5 min. The absorbance of the resulting supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of lipid peroxides, were quantified and expressed as total TBARS in terms of μmole g$^{-1}$ FW using an extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$.

**Activities of antioxidant enzymes:**

The activity of Superoxide dismutase (SOD) was assayed by the method of Beauchamp and Fridovich (1971) by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). The 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 15 mM methionine, 87 μM NBT, 2.4 μM riboflavin, 0.1 mM EDTA and 100 μL of enzyme extract. The test tubes were shaken and were illuminated with 15 W fluorescent lamp. The absorbance was taken at 560 nm. An illuminated blank without protein gave the maximum reduction of NBT, and therefore, the maximum absorbance at 560 nm. SOD activity is presented as absorbance of blank minus absorbance of sample, giving the total inhibition,
calculated per microgram protein. The activity of SOD was expressed as units mg\(^{-1}\) protein. One unit of activity is the amount of protein required to inhibit 50 % initial reduction of NBT under light.

The catalase (CAT) activity was measured by the method of Aebi (1984). The assay system comprised of 50 mM sodium phosphate (pH 7), 20 mM H\(_2\)O\(_2\), and 100 \(\mu\)L of enzyme extract in the final volume of 3 ml. Decrease in the absorbance was taken at 240 nm. The molar extinction coefficient of H\(_2\)O\(_2\) at 240 nm was taken as 0.04 cm\(^2\) \(\mu\)mol\(^{-1}\). Enzyme activity was expressed as units mg\(^{-1}\) protein.

Guaiacol peroxidase (GPX) activity was measured by the method of Chance and Maehly (1955). The reaction was initiated by addition of H\(_2\)O\(_2\) and change in optical density at 470 nm was measured at intervals of 10 s for 2 min. Activity was calculated using the extinction coefficient 26.6 mM\(^{-1}\) cm\(^{-1}\) for the oxidized tetruguaiacol polymer. Enzyme activity was expressed as units mg\(^{-1}\) protein.

**Metal estimation**

After harvesting the seedling, seedling were separated into roots, stems and leaves, after particular time of incubation for different experiments. These tissues were dried in oven at 80\(^\circ\)C till constant weight was reached. The dried plant samples were ground to fine powder with pestle and mortar. Weighed amount of powder was used for Cr, Cu and Cd estimation. These were digested with 3 ml of nitric acid and 1 ml of 70% perchloric acid on a hot plate under the hood. Digested sample solution was made to 10 ml volume with deionized water. Metal content in these samples was determined using Atomic Absorption Spectroscopy (Perkin Elmer 1100B). Metal content was calculated in mg per Kg of dry weight of tissue.