CHAPTER 3
MATERIALS AND METHODS

MATERIALS AND METHODS

The chapter describes the various chemicals, biological materials, instruments and equipments used for the study along with the detailed methodology of procedures and analysis used to obtain the results for the two species of *Portulaca* i.e. *P oleracea* and *P grandiflora.*

3.1 Source of plant material

P oleracea and *P grandiflora* were grown and maintained in Botanical Garden of the University and used as the source of explant. The plants were regularly watered without any application of fertilizers and pesticides. They were authenticated by Prof S.R Yadav, Department of Botany, Kolhapur University, Maharashtra. The prepared herbaria were deposited in The Maharaja Sayajirao University of Baroda Herbarium with voucher specimen numbers being AS1 for *P grandiflora* and AS2 for *P oleracea.*

- *P oleracea*, 4^{th}-5^{th} nodal leaves were utilized as an explant.
- *P grandiflora* nodal explants and young and mature leaf was utilized as a source of explants.

The plant material for metabolite studies were collected from the Botanical garden, thoroughly washed in running tap water, cut into small pieces and oven dried at 60ºC. Beetroot was purchased from the local market and utilized as a source of standard betalain.

3.2 Glassware

The following glassware were procured from Borosil India Limited and utilized for experimental work

- Beakers (100, 250 and 500 ml)
- Conical flasks (500 and 1000 ml)
- Culture tubes (25×150 mm/150 ml)
- Erlenmeyer flasks (150 and 250 ml)
- Glass tubes (5 ml) with Teflon lined screw caps
- Round bottom flask (100 and 250 ml)
- Test tubes

3.3 Chemicals

All the chemicals for Murashige and Skoog's medium (MS medium1962), sucrose, PGR's Agar-agar were procured from Sisco Research Laboratory Mumbai, India while $HgCl_2$ was purchased from Merck, India.

Fine chemicals like cytokinins and auxins were procured from Sisco Research Laboratory Mumbai India (SRL).

Solvents like Ethanol, Methanol, Propanol and Petroleum ether (40- 60ºC) were of analytical grade from SRL Mumbai India while Hexane was from Loba Chemi.

Supelco 37 component Fame mixture was purchased from Sigma Aldrich (metabolite studies).

Ethyl methane sulphonate utilized for mutation studies was purchased from SRL, Mumbai, India.

3.4 PLANT TISSUE CULTURE STUDIES

3.4.1 Instruments and Equipments

Several major and minor instruments and equipments were

(i) Instruments

- Microscope- Leica Research Microscope
- Oven- Hot air oven (Local make)
- Refrigerator- Kelvinator India
- Weighing balance- Sartorius balance (0.1 mg-100 gms) and Scasen balance (5-600 gms)

(ii) Equipments

- Horizontal Autoclave- Medicare Equipment Company**,** G.I.D.C Vadodara/ MAC Macro Scientific Works**.**
- Hot Plate-Jaymet Laboratory Equipments.
- Laminar Air Flow-Klenzaids/ Baroda Scientific Limited Vadodara India.
- Micro pipettes: Accupipet (100 µl) and Fisher (1ml and 5ml).
- pH Meter-Analabs Scientific Instruments Private Limited Vadodara India.

3.4.2 Preparation of Murashige and Skoog's Medium (1962)

The components of MS medium consist of macrosalts, microsalts, iron source and vitamins. The constituents of the stocks were added one by one only when the previous one had

dissolved completely in distill water and then stored at 4^0C. The constituents of MS medium are as follows:

Table 1: MS stock solutions and amount used for preparation of 1 l medium (Murashige and Skoog (1962)

Chemicals	Amount added (gm/l)	Volume of stock solution (ml/l)
Stock A [500ml] 10x		
NH_4NO_3	16.50	50
KNO_3	19.00	
$CaCl_2.2H_2O$	04.40	
$MgSO_4.7H_2O$	03.70	
KH_2PO_4	00.17	
Stock B [400ml] 200x		
KI	0.166	02
$CoCl_2$ $.2H_2O$	0.005	
H_3BO_3	1.240	
$Na_2MoO4.2H_2O$	0.050	
$MnSO_4.4H_2O$	4.460	
$ZnSO_4.7H_2O$	1.720	
$CuSO_4.5H_2O$	0.005	
Stock F [200ml] 100x		
$FeSO_4.7H_2O$	2.780	02
Na_2EDTA	3.730	
Stock-G [400ml] 200x		
Glycine	0.4	02
Nicotinic acid	0.1	
Thiamine HCl	0.1	
Pyridoxine HCl	0.1	
Myo-inositol	20	

- KH_2PO4 of Stock- A and Myo-inositol of Stock- G are added at time of medium preparation to avoid precipitation.

Initially the different stocks of MS medium were prepared as follows

3.4.2.1 Macro nutrients

It consists of macro salts of MS medium dissolved in distill water. The concentration of stock was kept at 10X and the volume was made upto 500 ml in distill water.

Table 2a: Macro stock of MS medium

MACRO SALTS	(X)mg/l	(10X)gm/l
$MgSO_4.7H_2O$	370	3.7
KNO_3	1900	19
NH_4NO_3	1650	16.5
$CaCl_2.2H_2O$	440	4.4
[500ml]		

KH_2PO_4 which is a part of macro salts tends to precipitate in the stock solution so it was added at the time of medium preparation.

3.4.2.2 Micro nutrients

Micro salts were dissolved in distill water and the strength of this stock solution was 200X in 400 ml of distill water.

Table 2b: Micro salts of MS medium

MICRO SALTS	(X)mg/l	(200X)gm/l
H_3BO_3	6.2	1.24
$MnSO_4.4H_2O$	22.3	4.46
$ZnSO_4.7H_2O$	8.6	1.72
$Na_2MoO_4.2H_2O$	0.25	0.05
$CuSO_4.5H_2O$	0.025	0.005
$CoCl_2.6H_2O$	0.025	0.005
KI	0.83	0.166
[400ml]		

3.4.2.3 Iron stock solution

The concentration of this stock solution was 100 X which was prepared in 200 ml of distill water. $FeSO_4$ and the chelating agent $Na_2EDTA.2H_2O$ were dissolved separately and boiled. Both the solutions were mixed in boiling condition, cooled at room temperature and stored at 4^0C.

Table 2c: Iron stock solution of MS medium

IRON STOCK	(X)mg/l	(100X)gm/l
$FeSO_4.7H_2O$	27.8	2.78
$Na_2EDTA.2H_2O$	37.3	3.78
[200ml]		

3.4.2.4 Vitamin stock solution

The stock was prepared in the concentration of 200X dissolved in 400 ml of distill water.

Table 2d: Vitamin stock solution of MS medium

VITAMIN STOCK	**(X)mg/l**	**(200X)gm/l**
Thiamine HCl	0.5	0.1
PyridoxinHCl	0.5	0.1
Nicotinic acid	0.5	0.1
Myoinositol	10	20
Glycine	2	0.4
		[400ml]

Myoinositol which is a component of vitamin stock precipitates in the stock solution so it was added at the time of medium preparation.

3.4.2.5 Stock solutions of PGR's

The MS medium was supplemented with different PGR's primarily cytokinins (BA and Kin) and auxins (IAA, IBA, NAA and 2,4D). These PGR's were prepared in mill molar stock and the volume drawn was according to the calculations in micromoles for the experiment. Cytokinins dissolved in 0.1 N HCl while auxins were dissolved in 0.1NNaOH and their final volume was made up with distilled water. Gibberellic acid was freshly prepared by dissolving in cold sterile water. All the PGR's were stored at 4ºC and utilized within one month of preparation.

The volume of PGR's used in the medium was calculated as follows:

Vol of PGR to be drawn from the stock= required concentration of PGR/ Strength of the stock prepared × Vol

3.4.2.6 Additives

The growth of the callus or shoots can be enhanced by the addition of other substances. These constituents are not a part of the defined medium and are used to increase the response. In the present studies coconut water was used as an additive.

- Coconut was procured from the local market and freshly used. It was boiled and sieved and on cooling was added to the medium.
- Silver nitrate was freshly prepared in cold water
- Phloroglucinol was freshly prepared in Ethanol

3.4.2.7 Modified macronutrient and micronutrient stock solution for shoot tip necrosis studies (*P grandiflora*)

The concentration of macro salts stock solution and micro salt stock solution of MS medium was modified for the study of shoot tip necrosis in *P grandiflora*. The usual concentration of Ca in the form of $CaCl_2$ is 3 mM in macro salts stock solution and Boron in the form of $H_3BO_3$is 0.1 mM in micro salt stock solution of MS medium. The concentration of Ca and B ions in MS medium was changed in the experiments for which a separate stock of both Ca and B ions was prepared. The rest of the constituent of both the macro and micro stock solution remained the same.

- **Macro salts of MS media with $CaCl_2$ as the source of Calcium**

A stock solution of $CaCl_2$ (100 mM) was prepared separately. Another stock of Macro salts was prepared which had all the constituents of macro salts of M S medium except $CaCl_2$.

- **Macro salts of MS media with Calcium gluconate as the source of Calcium**

In this stock solution $CaCl_2$ was replaced by Ca-gluconate as a source of Ca^{2+}. A stock solution of 100 mM Ca-gluconate was prepared separately. Macro salts of M S medium comprising of all the components of MS medium except $CaCl_2$ was also prepared separately.

- **Micro salts of MS media**

Similarly a stock solution of Boric acid (0.5 mM) and Stock B of MS medium devoid of Boron was prepared separately.

The volume of the stock solution was determined as:

$$\text{Volume of the stock (Ca/B as variable)} = \frac{\text{required concentration of the stock}}{\text{Concentration of stock}} \times \text{Vol required}$$

3.4.3 Carbon Source

Sucrose (30gm/l) was used as carbon source in MS medium.

3.4.4 Gelling agent

To solidify the medium agar agar (0.8% w/v) was used. The agar in the medium was brought to boiling and dispensed in culture vessels.

3.4.5 Medium preparation

The MS medium was prepared as follows:

- Addition of stocks of macro salts, micro salts, iron source and vitamins in known volume of distill water
- KH_2PO_4 and myoinositol were added one by one according to the requirement of the medium
- Sucrose (3%) was dissolved in the solution
- Addition of PGR's in µM as required and make up the final volume with distill water
- pH was set at 5.8 with the help of 0.1NHCl/1N NaOH
- Addition of agar (0.8% w/v).
- Medium was brought to boiling on a hot plate
- Dispensed in culture vessels
- Plugged with Teflon caps/cotton plugs and wrapped with paper.
- Medium, water, instruments and glassware required during inoculation process were autoclaved at 15 psi for 25 min.

3.4.5.1 Addition of GA_3 to the medium

Addition of freshly prepared GA_3 to the molten medium was done after sterilization of the medium as it is thermo labile.

3.4.6 Inoculation of the explant on the medium

The different stages of inoculation of the explant on the medium are as follows:

3.4.6.1 Surface sterilization of the explant

Healthy explants procured from the campus were transferred to a beaker, tightly covered with a cheese cloth and kept in running water for one hour. These explants were washed with a mild detergent (Labolene), rinsed with tap water several times and finally with distill water. Surface sterilization was done with 0.1% (w/v) $HgCl_2$for 3 min followed by rinsing in distill water 3-4 times to remove traces of $HgCl_2$.

3.4.6.2Procedure of Inoculation

All aseptic manipulations were carried out in laminar air flow cabinet which was swabbed with aqueous solution of Dettol followed by 70% alcohol. All the sterilized instruments, glassware, alcohol, cotton and medium were subjected to U.V light treatment for 45 min. The leaf explants were cut into $1cm^2$pieces and inoculated abaxially on the medium. The nodal explants were inoculated vertically on the medium stub. The culture tubes were again wrapped in sterilized paper and incubated in a culture room.

3.4.6.3 Incubation conditions

The culture flask/tubes after inoculation were incubated under cool white fluorescent 1200 mm, 18 Watt Phillips tube lights having photo period of 16/8 h light and dark cycle, at 25± 2ºC in a culture room

3.4.7 Regeneration from in vivo leaf explant

The regeneration studies were carried out utilizing leaf explants which were placed abxially on the MS medium fortified with 3% sucrose and following PGR's.

(a) Basal Medium (Control)

(b) Individual Cytokinin:

- BA/Kin (0.5, 2.5, 5, 10, 15 and 20µM) in *P oleracea*
- BA/Kin (0.5, 2.5, 5, 10) in *P grandiflora*

(c) Individual Auxin:

- IAA/NAA/2,4D (0.5, 2.5, 5 and 10µM) (Both the species)

(d) Combination of Cytokinins

- BA (0.5, 2.5, 5, 10, 15 and 20µM) + Kin (0.5, 2.5, 5, 10, 15 and 20µM) in *P oleracea*
- BA (0.5, 2.5, 5 and 10µM)+ Kin (0.5, 2.5, 5 and 10µM) in *P grandiflora*

(e) Combination of Cytokinin and Auxin

- BA/Kin (0.5, 2.5, 5, 10, 15 and 20µM) + IAA/NAA/2,4D (0.5, 2.5, 5, 10, 15 and 20µM) in *P oleracea*
- BA/Kin (0.5, 2.5, 5 and 10µM) + IAA/NAA/2,4D (0.5, 2.5, 5 and 10 µM) in *P grandiflora*

Periodical observations were taken and the data was recorded for a period of eight weeks. The different parameters evaluated for the study were:

$$\text{Mean number of shoots per leaf explants} = \frac{\text{Number of shoots}}{\text{Total number of explants}}$$

$$\text{Percent response for shoots from leaf explant} = \frac{\text{Number of explants forming shoots}}{\text{Total number of explants}} \times 100$$

3.4.8 Multiplication and elongation of in vitro shoots

The elongation and multiplication of in vitro shoots was tried in the following combinations.

(a) *P oleracea*

- Basal medium
- Coconut water – (0.5, 2.5, 5 and 10% v/v)

(b) *P grandiflora*

- Basal medium
- GA_3 – (2.5, 5 and 10µM)
- Coconut water

Ten shoot clusters on an optimized media for regeneration were taken for the study. The data for the number of shoot/cluster was recorded after 2 weeks and was calculated as follows.

$$\text{Number of shoot per cluster} = \frac{\text{Number of shoots}}{\text{Number of shoot clusters}}$$

3.4.9Regeneration from in vitro leaf explants

The in vitro shoots were obtained on an optimized regeneration medium from in vivo leaf explants. In vitro leaves were excised from the micro shoots aseptically, used as an explant and placed on an optimized media for regeneration in both *P oleracea* and *P grandiflora*.

3.4.10 Regeneration from nodal explant for *P grandiflora*

The effect of cytokinin was studied on nodal explants of *P grandiflora* and the combinations tested were as follows:

(a) Basal Media (Control)

(b) Cytokinin:

- BA/Kin (2, 4, 8 and 10µM)

(c) Combination of cytokinins

- BA (2, 4, 8 and 10µM)+Kin (2, 4, 8 and 10µM)

The number of replicates used for the study was 10 and the experiment was repeated twice. The different parameters for the study were as follows

$$\text{Number of shoots per explant} = \frac{\text{Total number of shoots}}{\text{Number of nodes}}$$

$$\text{Percent response for shoots from nodal explants} = \frac{\text{Number of shoots}}{\text{Number of nodes}} \times 100.$$

3.4.10.1 Study of shoot tip necrosis in *P grandiflora*

The MS media was supplemented with optimized concentrations of cytokinins for nodal cultures. The concentration of $CaCl_2$, Ca-gluconate and Boron was added as follows:

- $CaCl_2$ (3, 6, 12, 18, 24 and 30 mM)
- Ca-gluconate (3, 6, 12, 18, 24 and 30 mM)
- Boric acid (0.1, 0.2, 0.3 and 0.4 mM)

Observations for STN were taken for a period of 4 weeks and data for the number of shoots and percentage of necrosis was recorded as follows.

$$\text{Number of shoots per explant} = \frac{\text{Total number of shoots}}{\text{Number of nodes}}$$

$$\text{Percent Necrosis in nodal explant} = \frac{\text{Number of necrotic shoot}}{\text{Number of shoots}} \times 100$$

3.4.11 Rooting of micro shoots

The in vitro shoots were excised aseptically and given a pulse treatment (1 min) of 0.1% bavistin followed by dipping it in sterilized distill water to the remove traces of bavistin. The lower leaves from 2-3 nodes were removed and shoot were placed vertically with the support of filter paper bridge in the liquid medium.

(a) *P oleracea*

Rooting of in vitro shoots was done on liquid medium which was fortified with 1% sucrose and IBA (1-4 µM). Full and half strength media utilized and a control devoid of PGR's was maintained across all the concentrations.

(b) *P grandiflora*

The micro shoots obtained from *P grandiflora* leaf explants were rooted on half strength liquid MS media fortified with 1% sucrose and IBA (1 µM).

Data for the initiation of roots was taken for a period of 4 weeks with 10 replicates and the different parameters studied were:

$$\text{Mean number of roots per in vitro shoots} = \frac{\text{Number of roots}}{\text{Total number of in vitro plants}}$$

$$\text{Percent response for roots} = \frac{\text{Number of explants forming roots}}{\text{Number of explants}} \times 100$$

3.4.12 Establishment of callus cultures

3.4.12.1 *P oleracea*

Callus cultures were also established using leaf explants which were placed abxially on the MS medium supplemented with the following PGR's for the initiation of callus cultures.

(a) Individual Auxin:

- 2,4D (0.5-10µM)

(b) Combination of Cytokinin+ auxin:

- BA/Kin (0.5, 2.5, 5 and 10 µM) + 2,4D (0.5, 2.5, 5 and 10 µM)

Callus was evaluated with respect to colour, texture, and duration for initiation of callus. Growth of selected range of calluses was determined by taking their fresh weight and dry weight after 8 weeks. Dry weight of the callus was obtained by keeping it for drying at 40ºC in hot air oven till a constant weight was achieved. The number of replicates used for the study was 10 and the experiment was repeated twice. The different parameters studied in the experiment were as follows

$$\text{Percent response for callus} = \frac{\text{Number of explants forming callus}}{\text{Number of explants}} \times 100$$

Weight of the callus= Final weight of the callus – Initial weight of the callus

- **Callus growth index**

The callus was initiated on an optimized media and one gram callus was the size of the inoculums for the study of callus growth index. Six replicates were harvested every week and their fresh weight and dry weight was determined with which callus growth index was calculated.

$$\text{Callus growth index} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}}$$

3.4.12.2 *P grandiflora*

Callus from leaf explant was initiated on the following PGR's.

(a) Individual Auxin:

- 2,4D (0.5, 2.5, 5 and10µM)

(b) Combination of cytokinin + auxin:

- BA/Kin (0.5, 2.5, 5 and 10µM)+ 2,4D (0.5, 2.5, 5 and10µM)

The callus cultures were evaluated with respect to colour, texture, point of initiation and duration for initiation of callus.

3.4.13 Histology of regenerative leaf (Johnson 1940)

The explants growing on an optimized medium for both the plants were harvested weekly for a month. The harvested explants were fixed in FAA (Formaldehyde: Acetic acid: Alcohol) in the ratio of 2:1:10.The samples were dehydrated through ascending alcohol series and then transferred to xylene and embedded in paraffin wax (melting point 58º-60ºC). Serial sections of 10 – 15 µm were cut with a rotary microtome. The sections were stained in hematoxylene and eocine and mounted in DPX (Distreneplasticier-xylene). Photomicrographs were taken with Leica research microscope at 10X and 40X magnification.

3.5 METABOLITE STUDIES

In *P oleracea* primary metabolite fatty acids and within *P grandiflora* a secondary metabolite betalains were analyzed in the present study. The different materials used and the methodology adopted for the studies of the two metabolites are as follows:

3.5.1Instruments and Equipments

(i) Instruments

- Baby Centrifuge (Local make).
- Gas Chromatograph-Shimadzu Gas Chromatograph, GC-2014 equipped with G.C
- Solutions software. The column used in the instrument was Supelco Wax-10 (30m×0.25µm×0.25µm) with split/split less injector and detector used was Flame Ionization Detector (FID).
- Mass Spectrometer: Shimadzu, MS Model number GCMS-TQ8040. The capillary column used was 100% Polyethylene Glycol (Stabilwax- crossbondcarbowax60M length x 0.25mm ID x 0.25µm film thickness) and carrier gas was Helium.
- U.V Spectrophotometer- U.V- Vis Spectrophotometer, Perkin and Elmer, equipped with Lambda 25 software.
- Vortex- Genie 2, Bangalore India

(ii) Equipments

- Buchner Funnel
- Fine capillaries
- Micro pipettes: Accupipet (100 µl) and Fisher (1ml and 5ml)

- Nylon filter membrane: Pore size of 0.45µm and diameter of 13mm from Pall Corporation Mumbai
- Oven (Local make)
- Porcelain pestle and mortar
- Soxhlet apparatus
- Syringe: Exmire micro syringe from ITO Corporation Fuji Japan
- Water bath (Local make)
- Whatmann Filter paper- Schleicher and Schuell, Dassel, Germany

3.5.2 *P oleracea*- Fatty acids

3.5.2.1 Plant Material

The content of fatty acid was determined in in vivo shoot, stem, leaves, root, in vitro shoots, callus and in vitro shoots obtained after mutagen treatment. The in vivo samples were thoroughly washed in running water for one hour and left for air drying overnight followed by oven drying at 40ºC. The dried plant material was crushed to fine particles in a porcelain pestle and mortar and 10 gms of the dried powder were taken for extraction. The in vitro cultures were weighed and dried in the oven.

3.5.2.2 Preparation of chemicals

- **Sudan III dye**

Sudan III was freshly prepared as 0.1 gm of dye was dissolved in 50 ml of 70% alcohol and 50 ml of acetone, shaken and stored in dark colored bottles.

- **Methanolic BF_3**

BF_3 is a highly corrosive substance so it was added to chilled methanol to prepare a 10% solution and used for esterification of fatty acids.

3.5.2.3Preparation of sample for the extraction of fatty acids

The extraction was performed in a soxhlet apparatus and 10 gms of dried plant powder was placed inside the thick pad of cotton wool thimble and tapped gently. The whole apparatus was kept in a water bath and extraction was performed with 250 ml of petroleum ether. First extraction was done for 6 hours and the assembly was left as such overnight for cold extraction. The plant material was taken out from the thimble air dried and again crushed with a pestle and mortar and further extracted with 250 ml of petroleum ether for 4 h. The

extracts so obtained were pooled together in a 100 ml round bottom flask. Petroleum ether was distilled off in a distillation unit and the flask was oven dried for 15 minutes to remove traces of petroleum ether.

The fat obtained after oven drying was immediately subjected to analysis of fatty acids. Extraction of fatty acids from mutagen treated culture was done with 2 gms of the dry weight of the biomass. The samples were refluxed in 50 ml of petroleum ether for 2 h after which the solvent was distilled off. The rest of the procedure remained the same for the analysis of fatty acids.

3.5.2.4Test of fats

The oil obtained from soxhlet extraction was tested for the presence of fat. Two tests which are frequently used are Sudan III test and Emulsion test.

- **Sudan III Test**

Two ml of oil was taken in a test tube to which 2 ml of distill water was added. It was shaken and then 2-3 drops of Sudan III were added and shaken vigorously.

- **Emulsion Test**

Two ml of oil was taken in a test tube to which 2 ml of absolute alcohol was added and swirled vigorously after which 2 ml of cold water was added.

3.5.2.5 Esterification of Fatty acids (Weston et al. 2008)

The fat obtained after soxhlet extraction was esterified with 2 ml of 10% methanolic BF_3. The tubes were tightly screwed with Teflon lined screw cap and placed in water bath at 60ºC for 10 minutes. After cooling at room temperature the extract was vortex mixed for 30 min. To it 1 ml of water and 1 ml of hexane was added and again vortex mixed for 5 min. The tubes were centrifuged at 1000 rounds per minute for 3 min. After centrifugation the solution separated in two layers of which upper hexane layer was aspired and transferred to a bed of anhydrous sodium sulphate. The sodium sulphate adsorbed water molecules and 1µl of the sample was subjected to GC analysis.

A control was maintained throughout the experiments and the analysis for the sample was done in triplicates.

For the mutagen treated cultures the quantity of reagents was adjusted accordingly.

3.5.2.6 Fingerprinting of fatty acids

The standard used in the study was Supelco37 component FAME mixture, varied concentrations in dichloromethane. The FAME obtained was subjected to gas chromatography. Before injection the sample was passed through 45 µm nylon filter membrane. One µl of FAME was injected to gas chromatograph and the oven temperature program was as follows- The initial temperature of the column was 40°C held for 2 minutes then at 10°C/min ramp to 230°C and held for 10 minutes. The carrier gas hydrogen flow rate was 4.0 mL/min (0.61 bars) measured at 50° C. Split injection (1:10) was carried out at 230°C. The fatty acids were identified by comparing the retention time of the sample with that of the standards. Further confirmation of the fatty acids was done by GCMS analysis.

3.5.2.7 Mass Spectrometry for identification of fatty acids

The gas chromatograph was Shimadzu fitted with mass spectrometer of Shimadzu, MS Model number GCMS-TQ8040.The capillary column used was 100% Polyethylene Glycol (Stabilwax- crossbondcarbowax60M length x0.25mm ID x 0.25µm film thickness) and carrier gas was Helium. The injector port temperature was 220ºC and constant column pressure was maintained at 30kPa. The initial oven temperature was 80ºC and was increased at the rate of 10ºC/min to 230ºC finally, held for 33 min. The filament OFF time was 0.00-6.00 min and filament ON time was 6.10-50 min. The Electron Multiplier Voltage (EMV) mode was relative, Acquisition mode was Scan, mass scan range was 29-600m/z in an Electron Ionization (EI) mode, Electron energy 70eV, MSD interface temperature was 230ºC and MS ion source temperature was 200ºC.

Identification of the fatty acids was done by comparing the mass spectra of the fatty acids of the sample with the mass spectra of fatty acids obtained from National Institute of Standards and Technology (NIST) Library.

The different parameters studied were as follows:

(a) Estimation of total fat

For the estimation of total fats the weight of empty dried flask (W1) and the weight of flask+ Fat (W2) were taken.

Percentage amount of fat in all the samples was calculated as:

$$\text{Percentage of fat} = \frac{(W2-W1)}{S} \times 100$$

W2=Weight of the flask+ extracted fat

W1= Weight of the flask

S= Weight of the sample.

(b) Relative % of fatty acids by Gas chromatography and Mass Spectrometry

The fatty acids were identified by comparing their retention time with that of standard mixture of fatty acids and expressed in terms of relative percentage. The fatty acid analysis in GC studies was done in triplicates and the values were expressed as mean fatty acids percentage along with the standard error of the mean. The presence of these fatty acids was confirmed by GCMS studies and the Total Ion Chromatogram of all the samples and mass spectra of Linoleic acid and Linolenic acid was evaluated.

3.5.3 *P grandiflora*-Betalains

P grandiflora was analyzed for betalain pigment and the methodology adopted is discussed as follows:

3.5.3.1 Preparation of McIlvaine's Buffer

McIlvaine's buffer was prepared according to the table (McIlvaine 1921) for which a stock solution of 1 M $Na_2HPO_4$and 1 M Citric acid was prepared. For the present investigation a pH of 6 was utilized for the experiments.

3.5.3.2 Extraction of Betalains (Kugler et al. 2004)

Dry samples (10 gms) each of beet root and *P grandiflora* were taken and crushed to fine powder with pestle and mortar. Extraction was done with 100 ml of 60% methanol divided into two aliquots of 80 and 20 ml was prepared. First extraction was done with 80 ml of 60% methanol followed by second extraction of 20 ml in Buchner Funnel. Both the extracts were pooled together, concentrated in a water bath and taken for analysis.

3.5.3.3 Paper chromatography of Betalain

The mobile phase for paper chromatography of betalains was proponol: ethanol: water: acetic acid (6:7:6:1), prepared and poured in plastic boats which in turn were kept in a chromatography chamber for saturation. A chromatography paper of 25× 20 cms was cut and at 2 cms length the samples were loaded with the help of a fine capillary on the line. The spotted paper was hung in the chromatography chamber with the help of a glass rod and the bottom of the paper was dipped in the solvent system. The assembly was again kept in a glass

chamber and left for 1 h. The distance travelled by the solute and solvent was marked on paper and the Rf value was calculated.

$$\text{Rf value} = \frac{\text{Distance moved by the solute (ie dye or pigment)}}{\text{Distance moved by the solvent.}}$$

3.5.3.4 Spectrophotometric analysis

The filtrate obtained was subjected to Spectophotometric analysis. The filtrate was diluted with McIlvaine's buffer (pH 6) to obtain absorption values of $0.9 \leq A \leq 1.0$. Wave length (λ) scanwas given from 400-600 nm.

The different parameters studied were

The content of betalain was calculated by Spectrophotometric analysis.

- Dilution factor

Aliquot Volume

Final Volume= Aliquot volume+ Diluents volume

Aliquot- A measured sub volume of the original sample

Diluents-Material with which the sample is diluted

- Amount of betacyanin /betaxanthin present (Cai et al. 2005)

Where-A= Absorption value at the absorption maxima.

DF= Dilution factor

l= path length (1 cm) of the cuvette

MW= Molecular weight

€= molar extinction coefficient

Betacyanin

MW=550g/mol

= 60000 L/mol*cm in H_2O

λ= 540nm

Betaxanthin

MW=340g/mol

= 48000 L/mol*cm in H_2O

λ= 480nm

All the readings were taken in triplicates with UV-VIS Spectrophotometer which was equipped with lambda 25 software. The betalain content of whole plant and different parts of the in vivo plant (root, stem and leaves) was estimated.

3.6 MUTATION STUDIES

3.6.1 Instrument

Besides the Instruments utilized for Plant Tissue Culture studies and Metabolite studies other instrument employed was:

- Shaker:Orbitek

3.6.2 Preparation of Ethyl methane sulphonate (EMS)

Freshly prepared EMS was used by dissolving in sterile distill water under laminar hood and utilized as percentage.

3.6.3 Treatment of the in vitro leaves with EMS

Entire in vitro leaves were dipped in EMS solution and placed on the shaker for 1 h at a speed of 70 rpm for even treatment of the explants with the EMS solution. The concentrations tested for *P oleracea* were:

- 0.1-1% on an optimized media for regeneration
- 0.1-0.4% on an optimized media for callus induction

3.6.4 Estimation of LC50 Concentration

The first step in mutation study is to determine the LC50 dose for which the mortality of the treated cultures (0.1-1%) was calculated. The number of shoots/explant and callus weight of the treated cultures was determined. The morphological response was documented and the occurrence of variants was observed. The treated in vitro shoots/callus (2 gms) was analyzed for the fatty acid profiling in triplicates. The fatty acid profile of the variants was also determined.

The morphological response obtained on the EMS treated in vitro shoots was utilized for the estimation of LC50 concentration.

3.6.5 Inoculation of the EMS treated explants on an optimized media

Cultures were established with the EMS treated in vitro leaves on the optimized media for regeneration/callus induction in *P oleracea.*

3.6.6 Study of the EMS treated in vitro shoots and callus

The explants treated with different concentration of EMS were screened for the formation of in vitro shoots and callus cultures on an optimized media. The in vitro shoots and callus were then evaluated for their fatty acid content.

3.6.7 Histology of EMS treated in vitro leaf explant

Permanent slides of the mutagen treated in vitro leaf explant during 8^{th} week were prepared to evaluate the difference in cellular organization under the influence of different concentration of EMS (0.1-1%).

3.7 Statistical Analysis

For all the observation mean was calculated followed by standard error of the mean. Differences within the means were tested by one way analysis of variance (ANOVA) followed by Tukey's Honest Significant test where the value of $p \leq 0.05$. The LC50 concentration for mutation studies was determined by probit analysis on SPSS software while for other statistical analysis were done on MS Office Excel sheets.