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CHAPTER II

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CHAPTER II

MATERIALS AND METHODS

2.1 Materials:

2.1a Plant material:

Plant, Solanum xanthocarpum was used in this study. It was identified with Gamble's flora of Madras Presidency. The seeds were obtained from Gujarat Agricultural University, Anand, Gujarat and were grown in the botanical garden of M.S.University of Baroda in pots filled with garden soil on only supply of adequate amount of water.

2.1b Glassware:

All glassware were purchased from Durga Glass Works, Baroda. All of them were Borosilicate type. Depending upon the experiment they included Erlenmayer flasks, sugar tubes, petridishes of different diameters and test tubes. Fresh glassware were cleaned with dilute chromic acid solution (100 mg potassium dichromate dissolved in 1 litre of 1 N sulphuric acid) (Alexopoulose & Beneke,1955). Acid remains were removed by prolonged washing with tap water. Later they were washed sequentially with dilute solution of detergent (TeepolBDH),tap water and glass distilled water. Before use they were dried overnight in hot air oven.

2.1c Chemicals:

Mevinolin was a kind gift from Merck Sharp & Dohme Research Lab. U.S.A. Mevalonic acid and phytohormones were purchased from Sigma Chemical Co., U.S.A. 2-^{14}C 3-hydroxy 3 methyl glutaryl COA (50 μCi/m Mol) was purchased from New England Nuclear Corporation U.K. 2-^{14}C acetate was obtained from Bhabha Atomic Research Centre,Bombay. All other Chemicals used were of analytical grade purchased from the local market of Glindia India Ltd., B.D.H or S.D. fine Chemicals.

2.2 **Methods**

2.2a Composition and preparation of the media

The medium used throughout this study was MS medium (Murashige and Skooge 1962). Composition of the basal medium is given in the Tabale 2.1.

Basal medium was prepared by taking aliquotes out of the several concentrated stocks which were stored in the refrigerator at 4°C to 10°C and diluting to suitable volume with glass distilled water. Total 4 number of stock solutions were prepared as shown in the Table 2.1. Stocks of auxins and cytokinins were prepared by dissolving them in few drops of ethyl alcohol or 1 N HCl respectively and diluting to 100 ml so as to get a concentration of 1

Table 2.1 Murashige & Skoog (1962) medium composition and preparation

	Conc. of stock mg/l^{-1}	Volume of stock/ litre medium (ml)	Storage Temperature of stock solution
Major inorganic nutrients			
MSA			
NH_4NO_3	33000		
KNO_3	38000		
$CaCl_2 2H_2O$	8800	50	4°C
$MgSO_4.7H_2O$	7400		
$*KH_2PO_4$	3400		
Trace elements			
MSB			
KI	88		
H_3BO_3	620		
$MnSO_4H_2O$	2230		
$ZnSO_4.7H_2O$	860	10	4°C
$Na_2MoO_4.2H_2O$	25		
$CoSO_4.5H_2O$	2.5		

Table 2.1 (continued)

	Conc. of stock$_1$ mg/l^{-1}	Volume of stock per litre medium (ml)	Storage of stock solution
** Iron source			
MSF			
$FeSO_4.7H_2O$	5560	5	4°C
$Na_2EDTA.$	7460		
Carbon source			
Sucrose	30000		

* Myo-inositol and KH_2PO_4 were weighed and added separately at the time of media preparation.

**5.56 gms of $FeSO_4$ and 7.46 gms of Na_2 EDTA were weighed out in separate containers in about 200 ml of water, boiled separately and mixed with stirring while it was hot. Volume was made up to 1000 ml after cooling to room temperature.

millimolar. The aliquotes from the stocks were added in a sequential manner as given in Table 2.1. Addition of all supplements (Sugar, Casein hydrolysate & hormones) to the medium was done prior to making up the volume. The medium was adjusted to pH 5.8 using 0.1 N HCl or 0.1 N NaOH before the addition of 0.8% w/v difco bacto agar. Finally the medium was boiled to mix the agar and distributed into culture vessels uniformly.

2.2b Sterilization of the medium and culture vessels

All culture vessels containing media were sterilized in an autoclave at 120°C for 15 minutes under 15 lb pressure. A horizontal laminar flow cabinet (Klenzaids India) provided the asceptic chamber for tissue cultural manipulations . The work bench was wiped with 70% ethanol and the chamber was sterilized by U.V. germicidal lamp for 45 minutes. All tools were flame sterilized using 95 % ethanol on a bunsen burner.

2.2c Surface sterilization and inoculation of the explants

Young leaves from the seedlings were washed under running tap water for 1 hour. The leaves were then washed with distilled water. The surface sterilization was carried out in the laminar hood. For this the leaves were treated with 0.05 % w/v mercuric chloride for 2-3 minutes followed

by several rinses with sterile distilled water. The entire inoculation was done asceptically in the laminar hood. Leaf discs of 5 mm diameter were made from the sterilized leaves using sterile cork borrer. The leaf discs were transferred on to solid MS medium supplemented with 0.5 mgl^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D),0.1 mgl^{-1} Kinetin (Kn) and 250 mgl^{-1} Casein hydrolysate for induction of the callus cultures.

2.2d Initiation and maintenance of callus and cell cultures and plant regeneration from single cell clones

Callus Cultures: After inoculation of the leaf discs on the MSI medium (Table 2.2) the cultures were incubated for callus initiation at 25 $\pm$ 2°C in 16 h photoperiod under 1200 lux light intensity by day light fluorescent (Philips) tubes. Healthy white calli proliferated from the cut surface of the explants in 10-15 days. They were subcultured on fresh MSI medium many times at an interval of 15-20 days up to 6 months before they were used to initiate cell suspension cultures.

Cell suspension cultures: About 6 months old white friable calli were transferred to MS medium of similar composition but devoid of agar and incubated on a gyratory shaker at 120 rpm in the culture room 16 h photoperiod and 25 $\pm$ 2°C temperature. The medium is designated as MSL.

Table 2.2 Specifications of the media used in various experiments

Abreviation	
MSI Callus induction & Maintenance	MS salts + 0.5 mgl^{-1} 2,4-D, 0.1 mgl^{-1}kn, 250 mgl^{-1} CH, 0.8% w/v agar. pH 5.8 $\pm$ 0.02
MSL Cell culture	MS salts +0.5 mgl^{-1} 2,4-D,0.1 mgl^{-1} kn & 250 mgl^{-1} CH. pH 5.8 $\pm$ 0.02
MSP Cell plating	MS salts + 0.5 mgl^{-1} 2,4-D, 0.1 mgl^{-1} kn, 250 mgl^{-1} CH, 1.2% w/v agar. pH 5.8 + 0.02
MSS Shoot formation	MS salts + 2.25 mgl^{-1} BAP, 0.50 mgl^{-1} IAA, 0.8% w/v agar. pH 5.8
MSR rooting	MS salts 75% + 2 mgl^{-1} IBA, 0.55 mgl^{-1} NAA, 0.8% w/v agar. pH 5.8

Frequent subculturing were done at 9 days interval. At the beginning of each subculture the cell culture was filtered through 1 mm^2 mesh. 5 ml of the filtered suspension (200 to 300, mg fresh weight) was added to 30 ml of the fresh medium. After 6-9 subcultures fine pipetable cell suspension was obtained.

Growth analysis of cell suspension culture: About 6 months old cell culture of S. xanthocarpum was used for growth analysis. 5 ml of fine cell suspension (250-280 mg fr.wt.) was pipetted into 30 ml of fresh medium. Cultures were collected at 3 day interval till 15th day in triplicate and parameters of growth like fresh weight, dry wt., packed cell volume and cell number were determined as described by Reinert & Yeoman (1983). The growth analysis was performed several times.

Plant regeneration from single cell clones: Single cell cloning was performed essentially following Bergman's (1960) technique. About 10-12 months old cell culture of the late exponential phase (7-9 days old) was filtered successively through sterile stainless steel mesh of 600 μm^2,300 μm^2 and 120 μm^2 size to get fine cell suspension which consisted of single cells and small filaments of 2-4 cells as described

by Bhatt et al (1986). The cell suspension was diluted with sterile MSL medium to get a final density of 2×10^4 cells/ml.

2 ml of this cell suspension was pipetted into a 9 cm sterile glass petridish. Three ml of 1.2% w/v agar based medium (M S P, Table 2.2) containing 0.5 mgl^{-1} 2,4-D, 0.1 mgl^{-1} Kn, 250 mgl^{-1} casein hydrolysate (CH) and 3% w/v sucrose which was kept molten by maintaining at 38-42°C in water bath was added to the cell suspension in the petridish;mixed gently by swirling the petridish and allowed to solidify at room temperature. Thus $4x10^4$ plating units were plated per petridish. The petridish was sealed with parafilm and incubated in the culture room in an inverted position.

After 8 days of incubation many 6-10 celled colonies developed. The number of colonies were counted under an inverted microscope and plating efficiency was calculated according to the procedure described by Bhatt et al (1986). When the colonies reached a size of 2-3 mm diameter they were asceptically transferred to another petridish containing 15 ml fresh MS (MSI) medium. The calli that proliferated after 3-4 weeks were used for testing the morphogenetic potential.

Shoot differentiation was achieved on MS medium supplemented with indole-3-acetic acid (IAA) and Benzylaminopurine (BAP) at concentrations ranging from 0.1 to 10 μm under the same culture conditions as that described for callus growth. Regenerated shoots were transferred to dilute MS medium (75% MS salts + 25% water) supplemented with Indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA) at concentration of 10 μm and 0.1 μm respectively to induce roots.

Transplantation of the in vitro regenerated plants to the field: Tiny plantlets of 3.5 to 5 cm were transferred to pots containing sterilized mixtures of vermiculite and garden soil (1:1 ratio). The transferrred plants were acclamatized in the culture room environment for two weeks and were later transferred to the green house.

2.2e Biochemical techniques

(a) Steroid analyses of plant organs and cell culture of S. xanthocarpum: Determination of steroidal compounds were carried out from stem, leaf and berries of the seed derived plants grown in the University Garden. Steroids were estimated from cell cultures which were at lag phase, logphase and stationary phase of a particular batch.

2.2e (a.1) Extraction and analyses of free sterols, steryl esters and steryl glycosides. This was done using following procedure given in flow diagram.

1) Homogenize 1 gm plant material/cell culture in 3 ml of chloroform methanol (1:2 v/v)

↓

2) Filter this suspension (filterate collected)

↓

3) Reextract the residue in 3 ml chloroform methanol (1:2 v/v) and 0.8 ml of water.

↓

4) Filter through whatman No.1 filter paper under vacuum (filterate collected)

↓

5) Combine the filterate 1 and 2 and add 2.5 ml of pure chloroform and 2.9 ml of water and shake well in a separatory funnel. Allow two layers to separate.

↓

6) Separate the lower organic layer containing the lipids

↓

7) Evaporate the organic layer to dryness under reduced pressure and temperature (45°C)

8) Dissolve the dry lipids in 2 ml of Acetone:ethanol mixture (1:1 v/v)

9) Add 1 ml of 0.5% (w/v) digitonin in 50% aqueous ethanol

10) Free β-C-3 sterols of lipids will form insoluble complex with digitonin in this step. Allow the complex digitonides formation to complete overnight at room temperature. Centrifuge the mixture at 10,000 x g for 15 minutes for the recovery of digitonides.

12) Transfer the supernantant carefully using a pasteur pipette.

13) Wash the digitonides with 2 ml of acetone: diethyl ether mixture (1:2 v/v). Recover the washed digitonide by centrifuging at 10,000 x g for 10 minutes. Air dry the digitonides.

14) Dissolve the digitonides in 6 ml of glacial acetic acid.Add 4 ml of ferric chloride - sulphuric acid reagent.(Dissolve 2.5 gms of anhydrous ferric chloride in small amount of 85% ortho phosphoric acid dilute to 100 ml with the same acid. Dilute 8 ml of this concentrated stock to 100 ml using concentrated sulphuric acid to get the $FeCl_3H_2SO_4$ reagent).

15) Read O.D at 550 nm and calculate the quantum of sterol by using the standard graph (calibration curve). For the determination of steryl esters the supernatant obtained in step 12 was evaporated and the residues were collected in 0.75 ml of ethanolic potassium hydroxide and hydrolysed in boiling water bath (100°C) for 30 minutes in a sealed vial. Steryl esters were hydrolysed to free sterols and corresponding fatty acids during this process,. After cooling to room temperature the hydrolysate was neutralized to pH 7 using glacial acetic acid and the sterol fraction was extracted with 3 ml of pure chloroform. The amount of free sterol was then determined following the steps 7 to 15 of the procedure for free sterols

For the determination of the steryl glycosides the supernatant obtained in step 12 was evaporated to dryness in a vacuum evaporator at 45°C. The residue was collected in 2.5 ml of ethanolic sulphuric acid and hydrolysed for 11 hours in a tightly capped vial at 100°C in a boiling water bath. This reaction liberates free sterols from the glycosides. after cooling to room temperature the hydrolysate was neutralised with sodium hydroxide solution. Lipid fraction was extracted in 3 ml of pure chloroform and proceeded steps 7 to 15 as in the case of free sterols to

determine the amount of glycosides.

Preparation of the standard graph of cholesterol

A concentrated stock solution of cholesterol (1 mg ml^{-1}) was prepared in pure chloroform. Aliquotes of 10, 20, 40, 60 & 100 ul were pipetted out separately into conical centrifuge tubes and 2 ml of acetone : ethanol (1:1 v/v) mixture was added to each of the tubes. To this one ml of 0.5% (w/v) digitonin solution in 50% aqueous ethanol was added and left overnight at room temperaure for digitonide formation. Digitonide formed was collected and steps 7-15 was followed to prepare the standard graph.

(b) Extraction and analysis of steroidal alkaloid solasodine from plant organs and cell cultures:

The procedure of Chandler and Dodds (1983) which is a modification of procedure of Lancaster and Mann (1974) was used to determine the steroidal alkaloid contents of plant organs and cell cultures.

Plant organs (stem, leaves and berries) or cells were dried in an oven at 60-70°C to constant dry weight. The dried plant materials were ground to fine powder using mortar and pastle. 25 mg of the dried powder was refluxed in a sealed conical tube with 4 ml of 1 N hydrochloric acid for

two hours in boiling water bath and the hydrolysate was subsequently neutralised with 0.5 ml of 60% (w/v) sodium hydroxide. The liberated aglycone was extracted with 5 ml of chloroform, 2 ml aliquotes from this were pipetted out in to a separatory funnel and volume was made up to 5 ml with chloroform. 2.5 ml of 2 x 10^{-4} M bromothymole blue in borate buffer (.2 M boric acid + .05 M sodium borate, pH 8.0) was added and mixed for 10 seconds in a vortex stirrer. Care was taken to prevent the mixture being emulsified. Under this condition the aglycon formed a coloured complex with bromothymol blue. One ml of 0.02 N sodium hydroxide (prepared in methanol) was added to the complex. Optical density was determined using colorimeter (spectro colorimeter Model 103 (Systronics India) at 610 nm. Quantification of the alkaloid was done by comparing with the standard graph prepared using authentic solasodine as the standard.

Preparation of Solasodine standard graph:

Standard solasodine solution (1 mg ml^{-1}) was prepared in chloroform (ethanol free). Solasodine ranging from 10 to 80 ug was pipetted in test tubes and volume was made to 5 ml by adding chloroform. To this 2.5 ml of buffered solution of bromothymol blue was added and vortexed for 10 seconds. One ml of 0.02 N sodium hydroxide solution was added to the

coloured complex and OD was read at 610 nm to construct standard curve of solasodine. Solasodine recovery was 90%.

Qualitative analaysis of Solasodine:

500 mg of dry powdered plant material or cultured cells was refluxed with 40 ml of ethanolic hydrochloric acid in a sealed vial for 2 hours in boiling water bath and neutralised with 10 ml of 60% (w/v) sodium hydroxide. The liberated aglycone was partitioned into chloroform in a separatory funnel and the volume was reduced to 5 ml by evaporating under vacuum. A small volume of the concentrated aglycone was applied to previously activated (100°C 1 hr) T.L.C plate made of silica gel G. Authentic solasodine served as the standard. Plates were developed in n-Hexane: acetone mixture (4:1 v/v) and visualized by spraying with p-anisaldehyde reagent. Blue bands with Rf value corresponding to the standard solasodine appeared on heating the sprayed plates for 5 to 10 minutes at 110°C in a hot air oven.

2.3 Effects of mevinolin on the growth of seedlings, excised leaf discs and cell cultures of S.xanthocarpum

2.3a Seedling:

Seeds were collected from the plants grown in the botanical garden of the University and were germinated on

filter paper discs soaked in mevinolin solution of different concentrations in sealed petridishes under aseptic condition. Aqueous solution of mevinolin was prepared according to Kita et al (1984) (90 mg of mevinolin was dissolved in 1.8 ml of 95% v/v warm aqueous ethanol and was lactonised with 0.9 ml of 0.6N NaOH in 18 ml of water. It was adjusted to pH 8 with conc. HCl and the final concentration of the stock was made upto 4 mg/ml). During incubation temperature of 28-30°C and light intensity of 1200 lux was maintained. Effect of mevinolin was assayed as a function of inhibitions of germination, elongation growth, fresh and dry weight and accumulation of sterol and chlorophyll contents.

Determination of chlorophyll content was carried out following the procedure of Holden (1965). A known amount of the fresh tissue was ground in excess of 80% acetone in a mortar until all the colour was released into the solvent. The extract was filtered using a Buchner funnel and the residue was washed with acetone till it became free of green colour. The pooled filterate was made upto a known volume. The absorbance at 663 and 645 nm was measured using a 1 cm path length curet. Chlorophyll content was determined from the formula : total chlorophyll (mg/l) = 20.2 A 645 + 8.02 - A 663 where A = absorbance at 645 nm and 663 nm respectively.

Sterol content of the seedlings was determined as detailed earlier in section 2.2e(a.1)

2.3b Excised leaf disc:

Young leaves collected from the plants grown in the University botanical garden were surface sterilized as described earlier. Leaf discs of 10 mm diameter were cut out using cork borrer under aseptic condition. Leaf discs were cultured in sealed 9 cm petridishes on MS basal medium supplemented with 2% sucrose and varying concentrations of mevinolin (0.1 to 10 µM) under 1200 lux light intensity and temperature of 26±2°C. A time course study was carried out upto 120 hrs and optimal time point was determined for further experiments.

Effect of mevinolin on cultured leaf discs was assayed with respect to the inhibition in fresh wt.dry wt. and steroid accumulation. Determination of all parameters were carried out using the same procedure detailed elsewhere.

2.3c Cell suspension cultures:

Six to eight months old cell suspension culture maintained in batch culture was used for the experiment. Healthy cell culture in the late exponential phase (79 days) was used from a batch. Five ml of the filtered cell

suspension (250-280 mg ft.wt/5 ml) was pipetted out into 30 ml of fresh MS (MSL) medium containing different concentrations of mevinolin and was incubated on a gyratory shaker at 120 rpm under the same conditions of light and temperature used for maintenance of the cell cultures. Sets of 3 flasks were removed every 3rd day during the growth cycle and was analysed separately for the growth and biochemical parameters (sterols and alkaloids) as detailed earlier and the experiment was repeated.

2.3d Plated cells

Cell plating was carried out using 1.2 w/v agar based MS (MSP) medium supplemented with various concentrations of mevinolin (1 to 20 μM). After one week of plating micro colonies formed were counted microscopically and plating efficiency was calculated to determine the effect of mevinolin on colony formation of the plated cells.

2.4 Reversal of the mevinolin induced inhibition of growth and isoprenoid synthesis by exogenously supplied isoprenoid precursors to cultured leaf discs and cell cultures:

Reversal of the mevinolin induced inhibitions on metabolism and growth was done using the exogenous

intermediates of isoprenoid pathway (acetate, mevalonate, gibberellic acid (GA_3),squalene and cholesterol).

One millimolar stock solution of acetate was prepared by dissolving A.R. grade sodium acetate in distilled water. Mevalonic acid was prepared by dissolving mevalonic acid lactone (Sigma) in distilled water. Squalene stock was prepared in acetone and cholesterol stock was prepared in ethyl alcohol. Whenever the precursors were dissolved in organic solvents care was taken to see that the final concentration of the solvent in the medium did not exceed 0.05 % v/v. Moreover the controls were set up in such a way that the incubation medium contained a volume of the solvent equal to that in the experimental system. All experiments were set up in the same way as described to study the effects of mevinolin on seedlings, cultured leaf discs or cell cultures. Besdies mevinolin, the incubation medium contained various concentrations of the isoprenoid precursors. The reversal was assayed on the basis of changes in fresh and dry wt. and the changes in the isoprenoid contents of the seedlings, cultured leaf discs or cell culture as determined in the previous experiments.

2.5 Cellular selection for resistance to mevinolin and characterization of resistant cell lines

Selection of the cell line resistant to mevinolin was carried out through two different approaches. In first approach a single step selection was applied using cell plating and in second approach stepwise selection for resistance to mevinolin was attempted. At each step the conc. of mevinolin was increased for stepwise selection.

2.5a Selection of cell lines resistant to mevinolin:

Mevinolin resistant cell line was selected using the cell plating technique of Bergman (1960) as elaborated by Chaleffe (1983) and Maliga (1984). Approximately 4×10^4 live cells/plate were plated in MS medium (MSP) supplemented with a 1, 10, 15 & 20 µm concentration of mevinolin. After 8 days the plating efficiency was determined by counting the micro colonies formed under inverted microscope. The resistant colonies produced at concentrations that reduced the plating efficiency by 50%, 70% & 90% to control (lines were designated as L.D.50, L.D.70 and L.D.90 respectively) were aseptically transferred on to fresh MS (MSI) medium containing identical concentration of the drug and was maintained in similar medium till morphogenesis was attempted.

2.5b Stepwise selection of resistant line from cell cultures:

This approach in selection for resistant culture is analogous to that taken by Goldstein and his co-workers for Chinese hamster cell lines. Batch culture was setup using fine cell suspension prepared by sieving successively through 600 μm^2 300 μm^2, 120 μm^2 sterile stainless steel mesh. The density of the inoculum was kept minimal (150-200 mg cells/30 ml) to get best results. Six steps of selection was performed in this way to get resistant cell lines which could finally grow at 25 μM mevinolin. The resistant cell line obtained at low concentration (10 μM) was subjected to increasing concentrations (15,20,25) of the inhibitor in a sequential manner. After six rounds of selection (4 weeks in each conc.) with increasing concentrations of the drug a resistant cell line designated as JK^6 line was obtained which could grow in 25 μM of mevinolin. Wild type unselected cell lines cannot grow at this concentration. Further trials to grow these cell line on higher concentrations of the drug failed as the cells died of the toxicity exhibited by the drug.

2.5c Characterization of the resistant cell lines:

Characterization of the resistant cell line was done using following parameters (i) Ability to grow in the

presence of the drug, (ii) ability to synthesise steroids and steroidal alkaloid in presence of the drug mevinolin and (iii) the activity of HMG CoA reductase enzyme in the presence and absence of the drug.

The selected clones grown by regularly subculturing for 4 to 6 months in MS (MSL) medium supplemented with 10 um mevinolin at 9 day interval. Later on this cell line was transferred to MS (MSL) medium devoid of mevinolin for few culture passage and contents of sterols and steroidal alkaloid were determined. Unselected line which were grown on unselected medium (medium devoid of mevinolin) served as the control. This experiment was carried out to check the stability of the selected line. To get a better idea of their biosynthetic potential, both selected and unselected cell lines were grown for one passage in presence of the inhibitor and sterol and alkaloid contents were determined at the end of the passage. JK^6 cell line was also grown in presence and absence of mevinolin in comparison with unselected line and the steroids and alkaloid contents were determined. All the experiments were repeated.

In order to compare the rate of steroid synthesis in wild type and mevinolin resistant cell lines, $2\text{-}^{14}C$ incorporation studies using $2\text{-}^{14}C$ acetate was conducted. Acetate is reported to be a good precursor for its better

incorporation in to various isoprenoids. (Bhatt & Bhatt,1984;Chappel et al;1989). According to these authors and Subramani (1991) 3 day old cell cultures have the greatest rate of steroid synthesis.

Healthy, fine cell suspension (3 days old) derived from the wild (unselected) and the LD_{50} resistant line were transferred to the liquid medium supplemented with 2-^{14}C sodium acetate (100,000 cpm/ml) and incubated on a rotary shaker (120 rpm) for 30 minutes. Aliquotes of 2 ml were harvested at different time points,(10,20 & 30 min) washed with a dilute solution of non-radioactive sodium acetate to remove the unabsorbed radioactive acetate adhering to the cell wall through a buchner funnel fitted on a side arm flask connected to a vacuum pump and were immediately frozen to -20°C until they were used for biochemical estimations. Sterols were extracted as detailed earlier and separated on T.L.C. plates using chloroform: Methanol (95:5 v/v) as the solvent and were visualised using $FeCl_3$-sulfuric acid reagent. After heating for about 10 minutes in a hot air oven at 100°C bands developed corresponding to standard free sterols, steryl esters and steryl glycosides were cut, transferred to scintillation vials containing 10 ml toluene based Scintillation fluid (4 gms of 2,5' phenyl oxazole (PPO) and 400 mg (2,2'-phenylene-bis 5-phenyl oxazole

(POPOP) dissolved in 1000 ml of Scintillation grade toluene). Radioactivity (^{14}C) was counted on a L.K.B liquid Scintillation counter.

2.5d Assay of the HMG CoA reductase activity

3-Hydroxy 3-methyl glutaryl COA reductase is the gateway enzyme of isoprenoid pathway in animals(Goldstein & Brown,1990) and plants (Burden et al,1989). It catalyses the synthesis of mevalonic acid from 3-hydroxy 3-methyl glutaryl COA with the help of 2 mole of NADPH and ATP.

Studies using isoprenoid precursors as well as studies on the kinetics of this enzyme from Radish seedlings (Bach,1987) clearly indicate that mevinolin blocks the isoprenoid - synthesis by inhibiting this enzyme thereby not allowing the synthesis of first committed precursor of the pathway, mevalonic acid.

The enzyme assay was carried out according to the micro assay method of Chapell & Nabel (1987). Two gms fresh weight of tissue was homogenized in 2-5 volumes of 100 mM K-phosphate buffer (pH 7.0) containing 0.25 M sucrose, 50 mM KCl, 30 mM EDTA, 10 mM beta-mercaptoethanol with the addition of a small amount of insoluble poly vinyl pyrrolidone. The homogenate was filtered through glass wool

and centrifuged at 4000 g for 10' at 0°C in a (Eltek (RC 4100 D) refrigerated centrifuge. The supernatant was then used as the enzyme extract for assay of HMGR.

The activity of HMGR was assayed based on the conversion of ^{14}C HMG COA to ^{14}C mevanolic acid. The microassay system contained a total volume of 25 ul, which included 7 ul of enzyme extract, 3 n mol ^{14}C HMGCOA; (51 uci/mmol), 10 mM DTT & 4 n mol NADPH in 100mM K-phosphate buffer (pH 7). The reaction was started with the addition of 3 n mol of ^{14}C HMGCOA. The assay mix was incubated for 30' at 30°C and the reaction was stopped with the addition of 5 ul each of 6 N HCl and 5ul cold mevalonicacid (100 mg/ml) mevalonate lactone to induce formation of mevalonate lactone.

The mevalonate formed was separated from HMGCOA by silicagel TLC. Aliquotes of 5 ul were spotted on Silica gel plates and plates were developed in diethyl ether : acetone (3:1 v/v) and zones corresponding to authentic mevalonate (R_f 0.4-0.5) were scraped into Scintillation vials to determine radioactivity. Acid control was set up as a positive control. The activity is expressed as n mols of mevalonate formed h^{-1}. Calculation of the activity of HMGR was done based on the fact that a one : one conversion of HMGCOA to MVA takes place.Therefore if x cpm of HMGCOA = Y

moles of ^{14}C HMGCOA (based on the given specific activity of ^{14}C HMGCOA) it will be equal to Y moles of ^{14}C MVA. Specific activity of the enzyme is expressed as n moles of ^{14}C MVA formed/h/mg protein.

Protein level was determined by Bradford (1976) procedure. Protein in the enzyme preparation was precipitated using equal volume of chilled acetone for half an hour at 0°C temperature and pelleted by centrifuging at 10,000x g for 10 minutes at 0°C. The pellet was dissolved in minimal volume of 0.1 N NaOH solution and aliquotes of 100 ul were mixed with 900 ul of 0.1 N NaOH. To this 5 ml of Bradford reagent was added. The optical density was read at 595 nm. The standard curve was prepared using Bovine serum albumin (BSA).

Characterization of the regenerated S.xanthocarpum plants from the selected cell line

Characterization of the regenerated selected plants was carried out by leaf disc assay. Leaf discs (10 mm) were incubated on 10 µM mevinolin solution as described earlier for 72 h at 26±2°C and 16 h photoperiod. Leaf discs of the same size cultured under similar conditions collected from regenerated wild type cell line served as the control. Characteristics like growth (dry.wt) and sterol content were taken as the traits for resistance to mevinolin.