R E S U L T S

CHAPTER III

R E S U L T S

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Determination of growth kinetics of cell suspension culture.

Whole plant regeneration from the cell clones

Steroid and steroidal alkaloid contents of the S.xanthocarpum plant organs

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Leaf disc assay of variant regenerants

Analysis of steroidal contents of variant regenerants.

CHAPTER III

RESULTS

1. Establishment of Callus and Cell Cultures:

Young leaves of garden grown plants sterilized as detailed in chapter two and leaf discs of 10 mm diameter were transferred to agar based MS medium supplimented with 0.5 mg/l 2,4-D, 0.1 mg/l kn, 250 mg/l casein hydrolysate, 3% w/v sucrose for induction of callus. White slightly friable callus formed in 10-15 days of incubation in this medium. Friability of the callus increased with further sub-culture (plate-1).

For the initiation of cell culture 200 to 300 mg of this friable callus was transferred to liquid medium of same composition as that used for callus initiation. Very fine suspension of isolated cells and small clumps with 80-90 % viability was obtained after 3-4 sub-cultures performed at 15 days interval.

2. Determination of the growth kinetics of cell suspension culture:

Different parameters of growth viz., fresh wt., packed

Plate I Induction of callus from the leaf discs of S.xanthocarpum when cultured on MS medium supplemented with 0.5 ppm 2,4-D and 0.1 ppm Kn.


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cell volume and cell number per flask were investigated to study the growth kinetics. As shown in the Fig.1 fresh wt. and dry wt. showed characteristic sigmoid pattern of growth with a lag phase of 6 days and thereafter a rapid growth period commenced till 12th day and entered into the stationary phase thereafter. The doubling time for packed cell volume, dry weight and fresh weight was 17.14, 17.56 and 17.22 hours as shown in the table 1.

Table 1. Growth response of cell culture of Solanum xanthocarpum

Parameters used	Mean doubling time (h)
1. Fresh weight	17.14
2. Dry weight	17.56
3. Packed cell volume	17.22

Growth of suspension culture on agar plates and plating efficiency:

Plating of isolated cells and small aggregates (3-5 cells) on 0.8 % w/v agar based MS medium supplemented with 250 mgl^{-1} casein hydrolysate, 0.5 mgl^{-1} 2,4-D and 0.1 mgl^{-1} kinetin resulted into 70-75% plating efficiency when plated

Fig.1 Growth kinetics of cell cultures of S.xanthocarpum (o-----o) Fr.wt. (•——•) dr.wt.

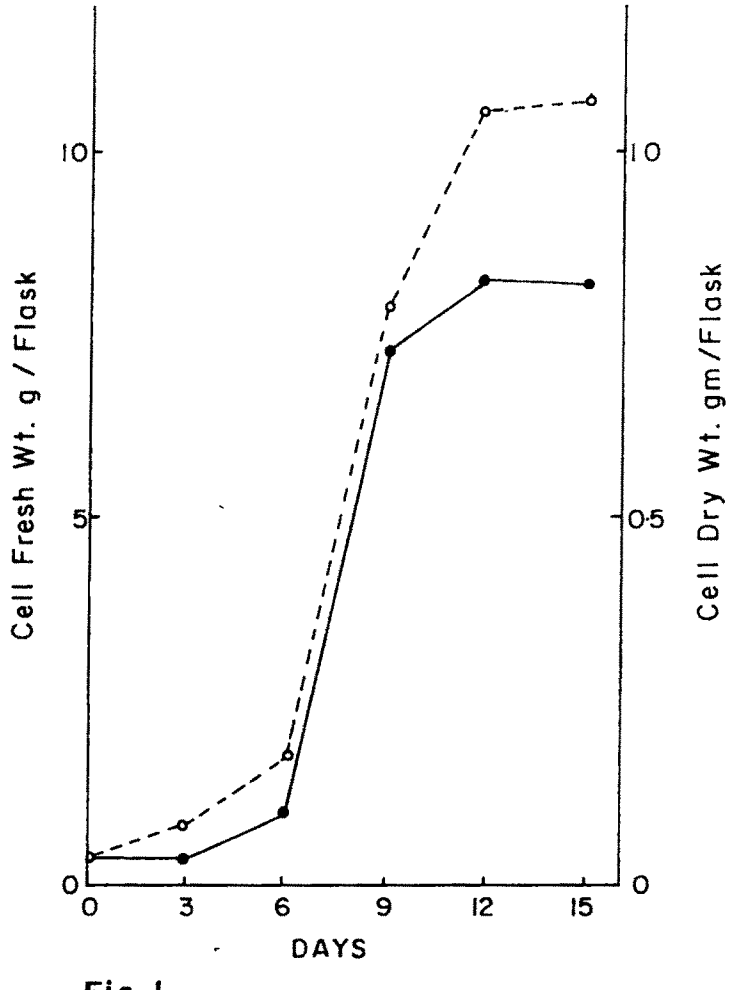

Cell Fresh Wt. g / Flask
Cell Dry Wt. gm/Flask
10
5
0
1·0
0·5
0
0
3
6
9
12
15
DAYS

Fig. 1

at a density of 4 x 10^4 cells per 9 cm petridish containing 3 ml of the medium. Earlier Subramani (1991) indicated that the change in the cell density or change in the culture media or addition of different addenda could not raise the plating efficiency above 75 %.

3. Whole plant regeneration from the cell clones:

Callus pieces derived from plated cells (3-5 mm^2) were transferred on to a medium supplimented with various concentrations of phytohormones, viz.,BAP, IBA, NAA and IAA. Shoot regeneration was obtained on various combinations of phytohormones but maximal frequency of shoot development was obtained in a medium supplemented with 0.1 µm IAA and 8 µm BAP (Table 2) (Plate 2a). Shoots developed within 7 to 10 days of incubation on such a medium. Roots were induced to form at the cut ends of the shoots on half strength MS medium in the wild type but addition of 10 µm of IBA and 0.1 µm NAA was required to induce roots on the shoots derived from clones of the mevinolin resistant cell line (Plate 2b & Plate 3)

Steroid and steroidal alkaloid contents of the S.xanthocarpum plant organs.

Steroids viz., free sterols, steryl esters, and steryl glycosides and steroidal alkaloid (solasodine) contents were

Plate 2.a.i Development of shoots from the wild type callus of S.xanthocarpum .

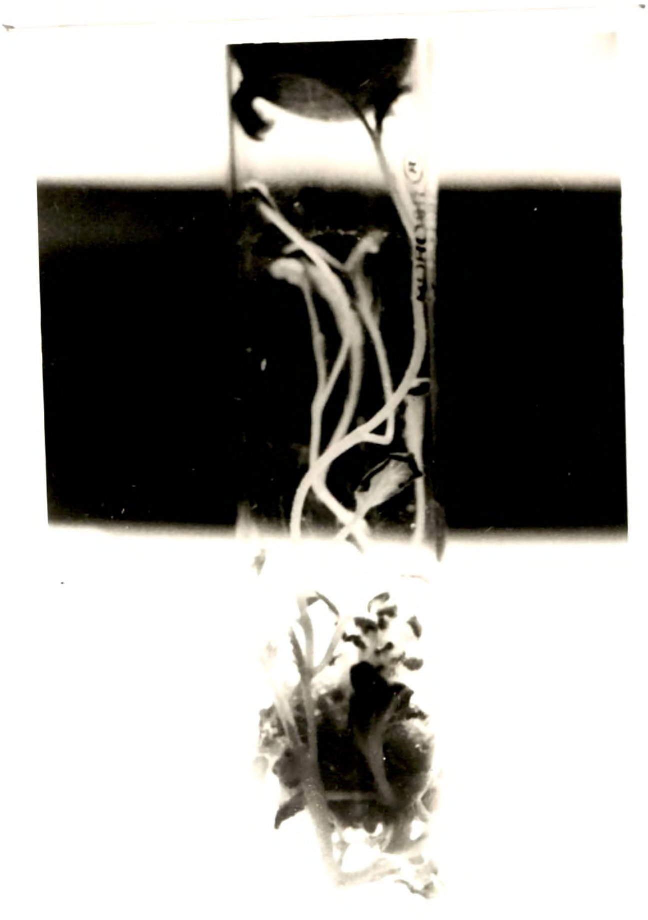


Plate 2.a.ii & iii Greening & development of shoots from the selected mevinolin resistant cell lines of *S. xanthocarpum*

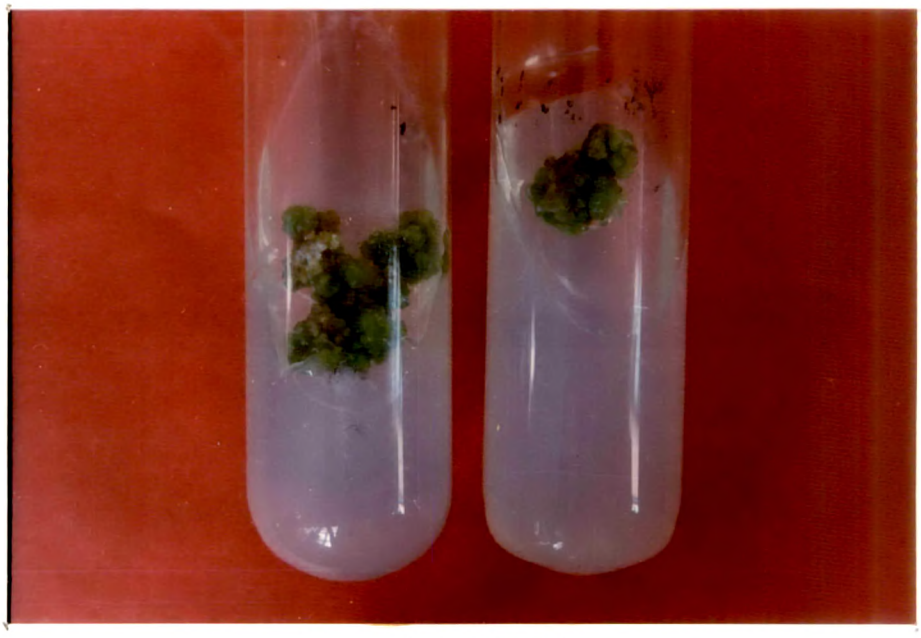


Plate 2a. ii

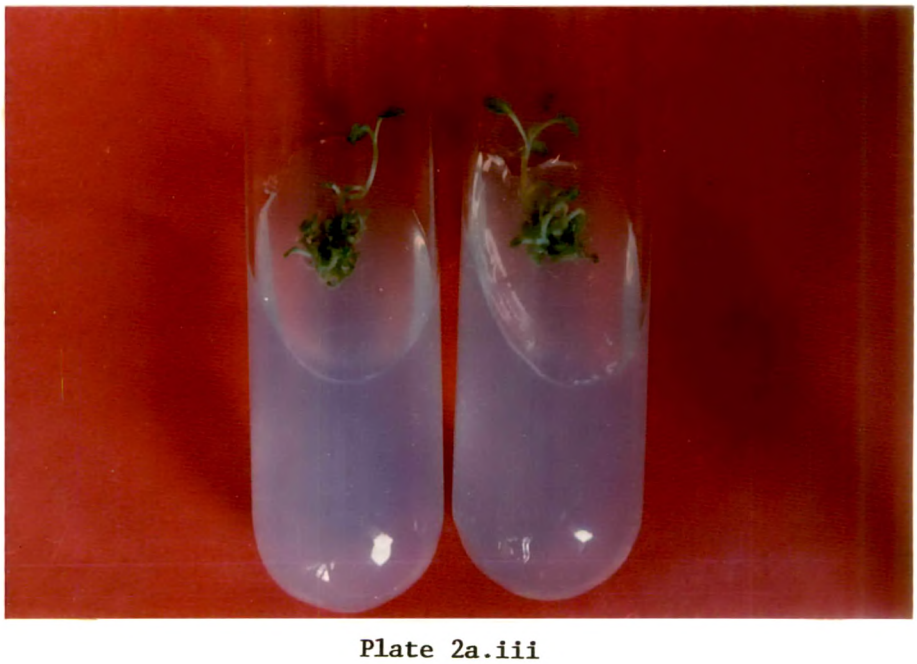


Plate 2a.iii

Plate 2.b. Rooting of the *in vitro* derived shoots of *S.xanthocarpum*.

Plate 3 Regenerated mevinolin resistant *Solanum xanthocarpum* in the pot.

Table 2

Effect of IBA and NAA on rooting of the *in vitro* developed shoots of *S.xanthocarpum* raised from the selected cell lines.

IBA µM	NAA µM	% of shoot rooted
1	0.1	10 - 20
2	0.1	25 - 30
4	0.1	40 - 50
8	0.1	60 - 70
10	0.1	85 - 95

determined in various plant organs like leaves, stem and berries (Table 3). Maximum amount of free sterol (181.3 µg/g fr.wt.) and steryl glycosides (146.5 µg/g fr.wt.) was found in immature leaves, but highest amount of steryl ester was found in the young stem portion (117 µg/g fr.wt.) Maximum amount of steroidal alkaloid (solasodine) (3.66mg/g dry wt.) was found in the young berries when compared to other plant parts.

Changes in Free sterol, steryl ester, steryl glycosides and solasodine levels in a batch cell cultures:

Levels of free sterol, steryl ester, steryl glycosides and Solasodine contents during the growth cycle of a batch culture were measured. The free sterol level fluctuated during the growth cycle where as the steryl ester level remained low on 3rd day but increased thereafter till stationary phase was reached. In the case of steryl glycosides the level remained low on 9th day and increased thereafter rapidly. The content of steroidal alkaloid solasodine was high during the early (3rd day) to the mid log phase (9th day) of growth cycle (Fig.2).

1. Effects of mevinolin on germination and growth of the seedlings of S. xanthocarpum

Sterilized seeds of S.xanthocarpum were germinated on wet filter paper discs under aseptic conditions in the

Table 3. Analyses of β-C-3 sterols, steryl ester and glycosides and alkaloid of plant organs of Solanum xanthocarpum

	Sterols µg/gm fr. wt.			Steroidal alkaloid (mg/g dry. wt.)
	Free sterol	Steryl ester	Steryl glycoside	
Immature Berries	96	64.5	75.5	3.66
Immature Leaves	181.3	86.3	146.5	2.13
Immature Stem	85	117	39.6	1.86

Fi.2 Changes in the steroidal contents of cell cultures of Solanum during a growth cycle. Free sterols (●——●) steryl esters (△---△) steryl glycosides (▲—▲) and solasodine (○-----○).

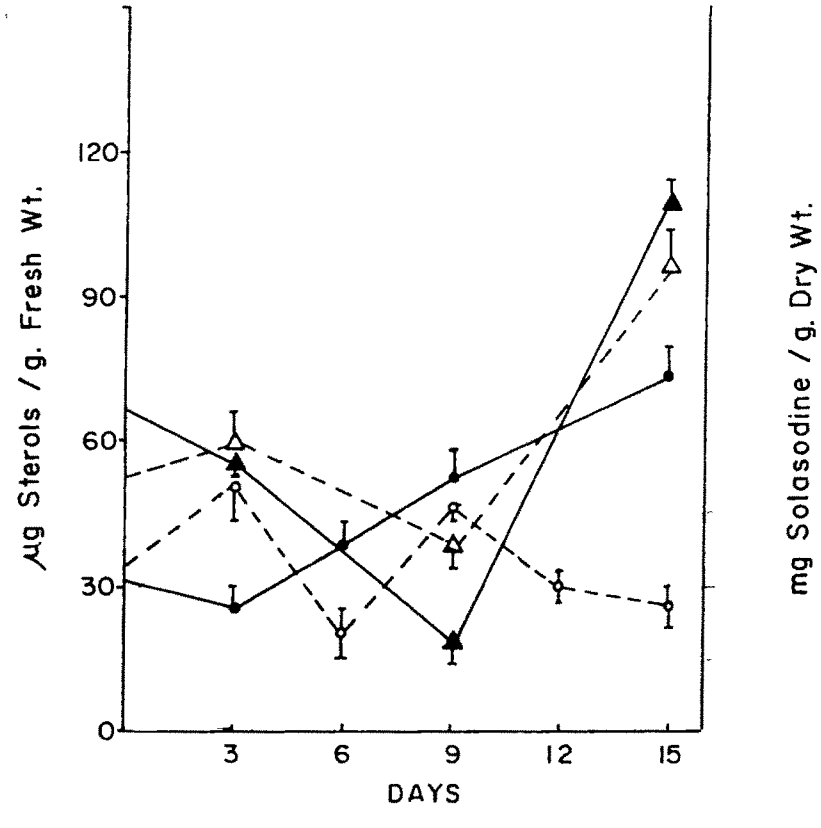

120
90
60
30
0
3
6
9
12
15
DAYS
µg Sterols / g. Fresh Wt.
mg Solasodine / g. Dry Wt.

Fig. 2

presence of different concentrations of aqueous mevinolin, varying from 1 to 100 μm. The data obtained did not show any inhibitory effect of mevinolin on germination of seeds.

In order to study the effects of mevinolin on steroid biosynthesis and the growth of the seedlings, seeds were germinated and grown in the presence of different concentrations of mevinolin (1-100 μM), under 16 hr photoperiod and 26 ± 2°C temperature. The results indicated that the inhibition of the root growth was greater than the shoot growth. Analysis of the pigment content and sterol content revealed that the sterol biosynthesis was inhibited to more than 50% (Table 4 & 5).

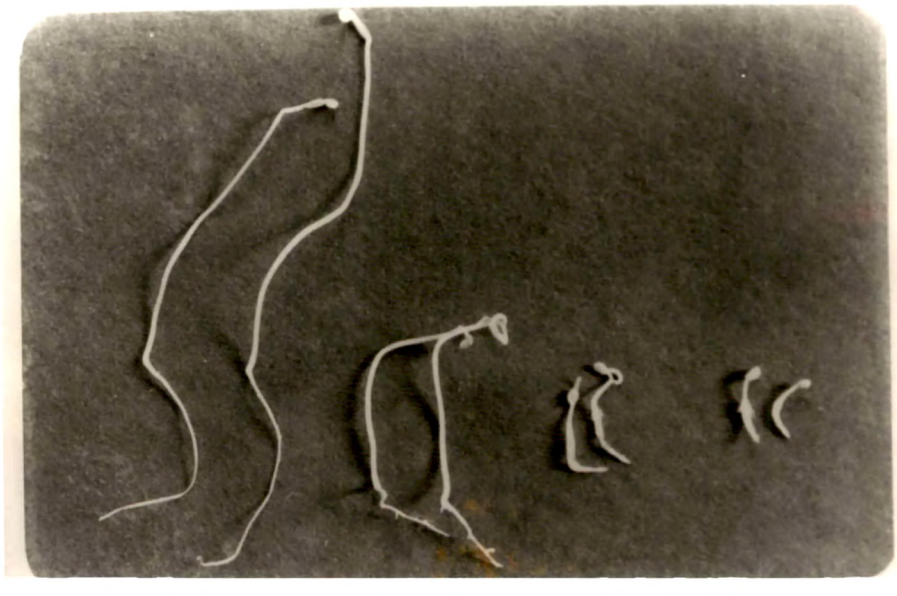
However there was considerable difference in the degree of inhibition of growth of the dark and light grown seedlings. Root growth was inhibited to a greater extent when seedlings were grown in the darkness but shoot growth inhibition was not that pronounced with regard to the light conditions (Table 4 & 5, Fig. 3 & 4, Plate 4)

2) Effects of mevinolin on cultured leaf discs:

Sterile leaf discs of 10 mm diameter were cut from young leaves of garden grown plants and were cultured on MS basal medium supplemented with 2% sucrose and different concentrations of mevinolin 0.01 - 10 μM).

Plate 4. Effect of mevinolin on seedlings of S. xanthocarpum. Photographed after 10 days of mevinolin treatment.

a) Control b) +25 μM mevinolin c)+ 50 μM mevinolin d) + 100 μM mevinolin.



a b c d

Table 4 : Effects of mevinolin on growth & sterol metabolism of etiolated seedlings of *S. xanthocarpum* Data collected after 10 days of germination.

Treatment	Shoot length cm±S.E	Root length cm±S.E	Fresh weight mg/seedling ±S.E	Dry wt./25 seedlings(mg) ± S.E	Free sterol µg/gm.fr.wt. ± S.E
Control	5.31 ± 0.27 a	3.96 ± 0.12 a	15.69±0.45 a	3.56±0.14 a	133.22±7.32 a
10 µM mevinolin	3.62 ± 0.18 b	1.23 ± 0.08 b	11.87±0.64 b	2.61±0.11 b	66.00±2.93 b
25 µM mevinolin	3.54 ± 0.12 b	1.09 ± 0.08 b	10.41±0.48 b	2.43±0.88 b	66.00±2.78 b
50 µM mevinolin	2.42 ± 0.20 c	0.49 ± 0.04 c	8.29±0.26 c	2.00±0.57 b	75.66±3.18 b
100 µM mevinolin	2.09 ± 0.11 c	0.37 ± 0.03 c	7.47±0.24 c	1.68±0.066c	64.00±2.26 c

Values with different letters indicate significant difference at 5% level.

Table 5 : Effect of mevinolin on light grown seedlings of S.xanthocarpum. Data collected after 10 days of germination

Treatment	Root length (mm)±S.E	Shoot length (mm)±S.E	Fr.wt.mg/ seedling ±S.E	Dr.wt.mg/ 25 seedlings ± S.E	Free sterol µg/g.fr.wt. ± S.E
Control	37.0 ± 1.5 a	19.65 ± 0.07 a	11.22±0.35 a	2.43±0.08 a	122.66±4.06 a
10 µM mevinolin	12.7 ± 1.5 b	13.5 ± 3.1 b	8.46±0.25 b	1.65±0.14 b	88.66±3.23 b
25 µM mevinolin	8.1 ± 0.5 c	11.10 ± 3.10 b	7.63±0.22 b	1.4 ±0.015 b	84.66±4.62 b
50 µM mevinolin	3.8 ± 0.5 d	8.8 ± 0.6 c	6.47±0.26 c	1.26±0.014 b	64.58±3.68 c
100 µM mevinolin	3.05 ± 0.02 d	7.2 ± 0.04 c	5.58±0.25 c	1.16±0.012 c	56.46±2.36 c

Values with different letters indicate significant difference at 5% level

Fig.3 Effect of mevinolin on the dark grown seedlings of Solanum. Data collected after 10 days of growth. Shoot length (▲——▲), root length (□----□), fresh wt. (●——●), dry wt. (○----○) and free sterols (△---△).

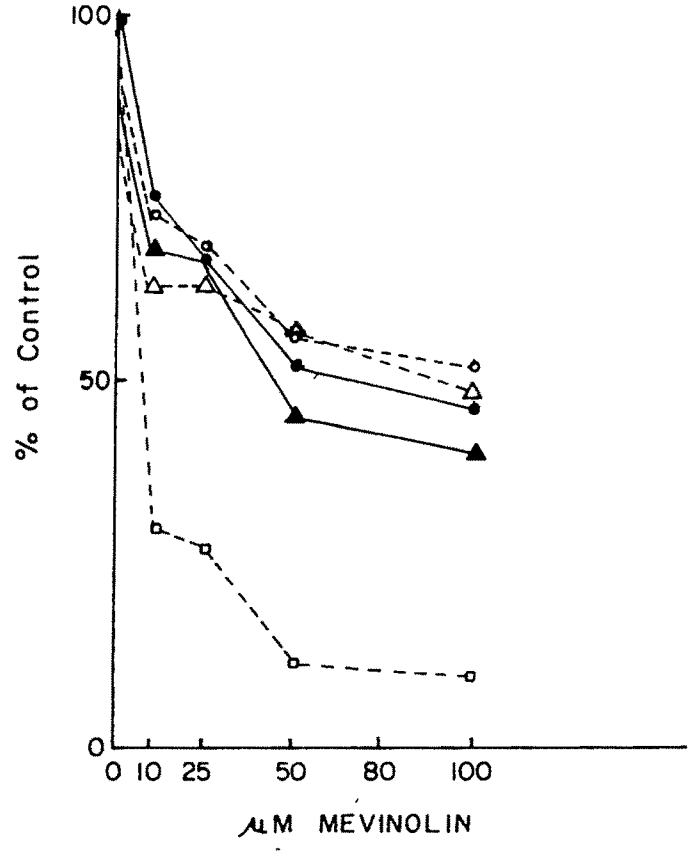


Fig. 3

Fig.4 Effect of mevinolin on light grown seedlings of Solanum. Data collected after 10 days of growth. Shoot length (▲—▲), Root length (□----□), Fr.wt.(○----○), dry wt.(●----●) and free sterols (△----△).

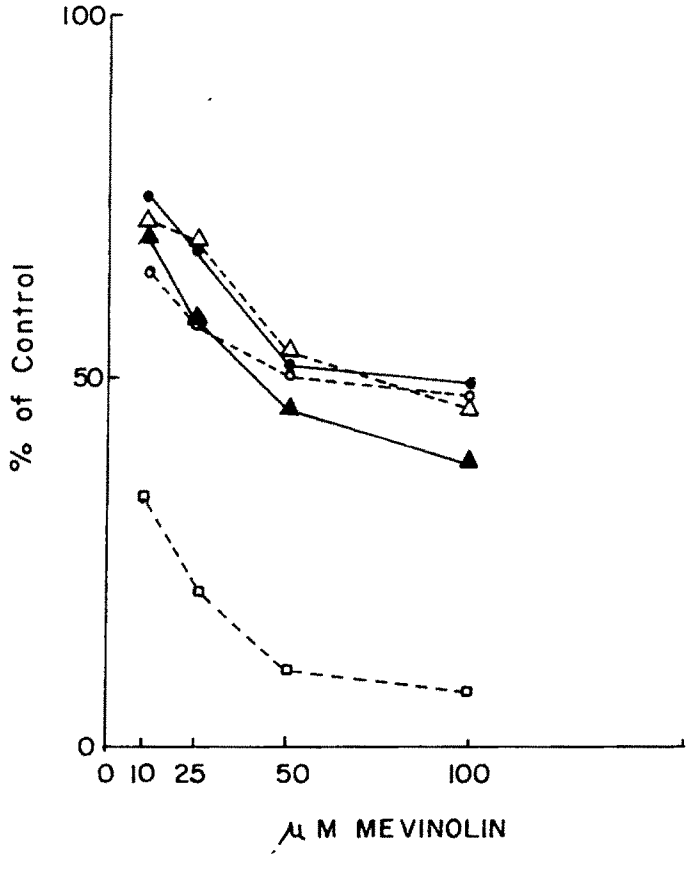


Fig. 4

Quantitative determinations of the contents of free sterols (β-C-3) fresh and dry weight and pigments (Chlorophyll and carotenoids) were carried out. Sterol content was reduced more than 50% by 10 μM mevinolin and growth was severely affected while there was not much influence on the pigment accumulation (Table 6&7. Fig.5 & 6).

3) Effects of mevinolin on batch cell cutlure:

6-8 months old batch cell cultures was grown for one passage in presence of different concentrations of mevinolin ranging from 1 μM to 50 μM.. The inhibitory effects of mevinolin was calculated on the basis of difference in growth and steroid accumulation. Growth and steroid accumulation of the cell culture was adversely affected in a dose dependent manner. Concentration of mevinolin at 25 μM showed almost 50 percent (L.D.50) inhibition of growth and steroid accumulation of the cell cultures and the 50 μM concentration was totally lethal (Fig.7 & 8).

4) Effects of mevinolin on plated cells:

Cells derived from batch suspension culture (7-9 day old) were plated on 9 cm plates on agar based MS medium containing different concentrations of mevinolin ranging from 0.1 to 20 μM. The inhibitory effect of the drug was quantified by decrease in the plating efficiency (Fig.9).

Plate 5 Effect of mevinolin on plated cells

a) Control b) +0.1 μM mevinolin,

c) + 1 μM mevinolin d) + 10 μM mevinolin

e) + 20 μM mevinolin.

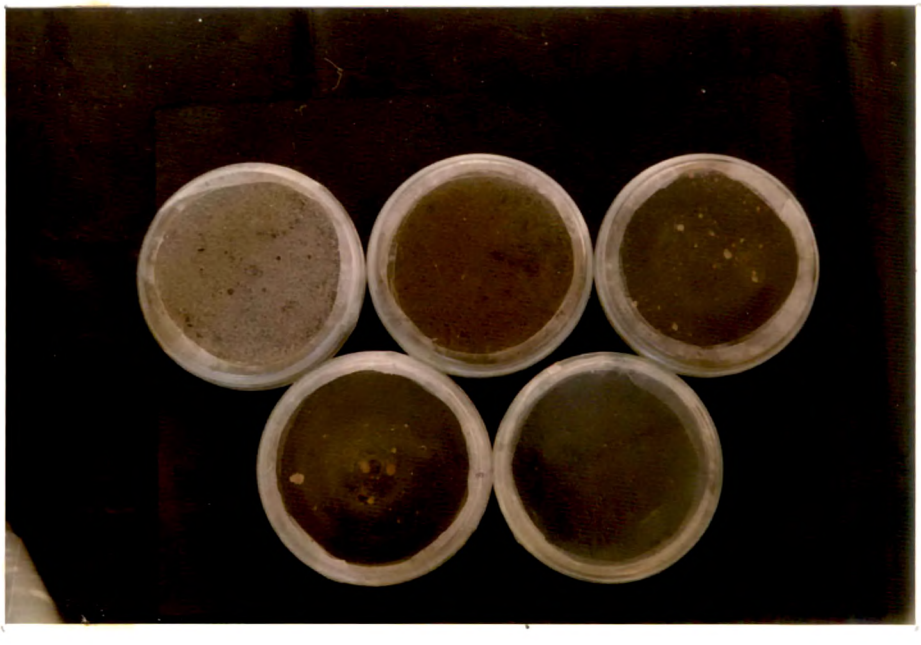


Table 6 : Effects of mevinolin on cultured leaf discs of S. xanthocarpum. Incubation was carried out for 72 h under 16-8 photoperiod, 25 ± 2°C temperature.

Treatment	Dry weight (mg)/leaf ±S.E	Chlorophyll (mg/gm.fr.wt) ±S.E.	Free sterols (µg/gm.fr.wt.) ±S.E
Control	6.02±0.37 a	0.80±0.066 a	149.11±17.59 a
0.01 µM mevinolin	6.62±0.80 a	0.75±0.075 a	111.79±20.99 b
0.1 µM mevinolin	6.32±0.48 a	0.710±0.081a	97.6 ± 9.34 b
1 µM mevinolin	5.21±0.19 b	0.68±0.043 a	83.46± 6.87 b
10 µM mevinolin	3.75±0.51 c	0.66 ±0.017b	62.15± 5.16 c

Values with different letters indicate significant difference at 5% level

Table 7: Effect of 10 μM mevinolin on growth and sterol and pigment accumulation of cultured leaf discs of Solanum xanthocarpum

Time (hrs)	Dry wt/10 mm leaf discs ± S.E		Free sterols μg/gm fr.wt.± S.E		Carotenoid content mg/gm fr.wt.± S.E		Chlorophyll content mg/gm fr.wt.± S.E	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
24	6.01±0.009[a]	6.0±0.003[a]	144±8.4[a]	139±5.8[a]	0.92±0.07[a]	0.90±0.02[a]	0.71±0.03[a]	0.63±0.037[a]
48	6.20±0.043[a]	5.3±0.012[a]	162±7.4[a]	124±13.3[a]	0.92±0.06[a]	0.73±0.06[b]	0.65±0.04[a]	0.57±0.04
72	6.8 ±0.03[a]	3.3±0.013[b]	189±6.6[b]	115±3.64[b]	1.00±0.1 [b]	0.89±0.03[a]	0.71±0.02[a]	0.52±0.07

Fig.5 Effect of mevinolin on cultured 10 mm leaf discs of solanum. Data collected after 72 hours of incubation. Dry wt. (▲——▲), Chlorophyll (●——●) and free sterols (○- - -○).

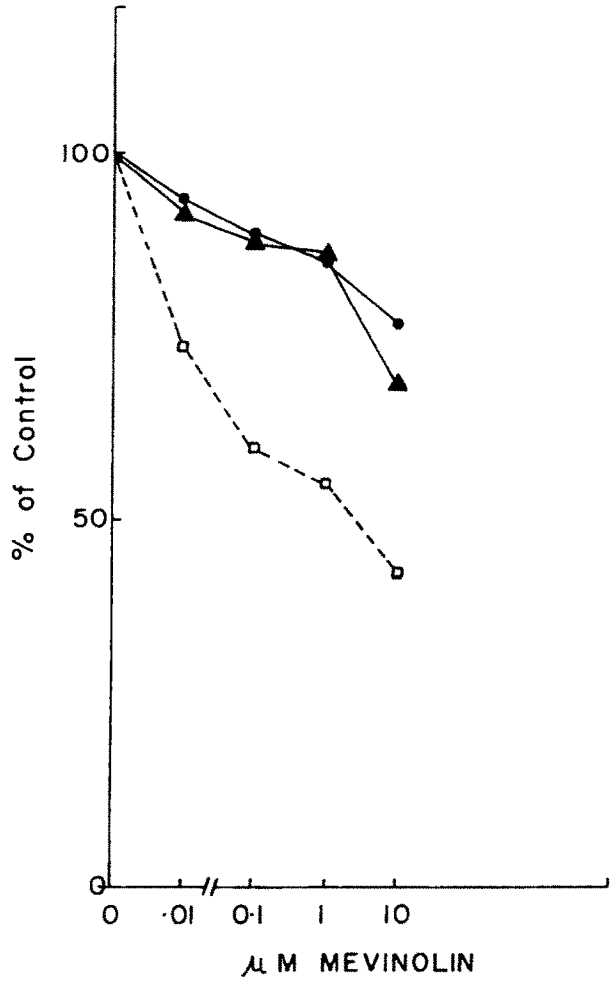
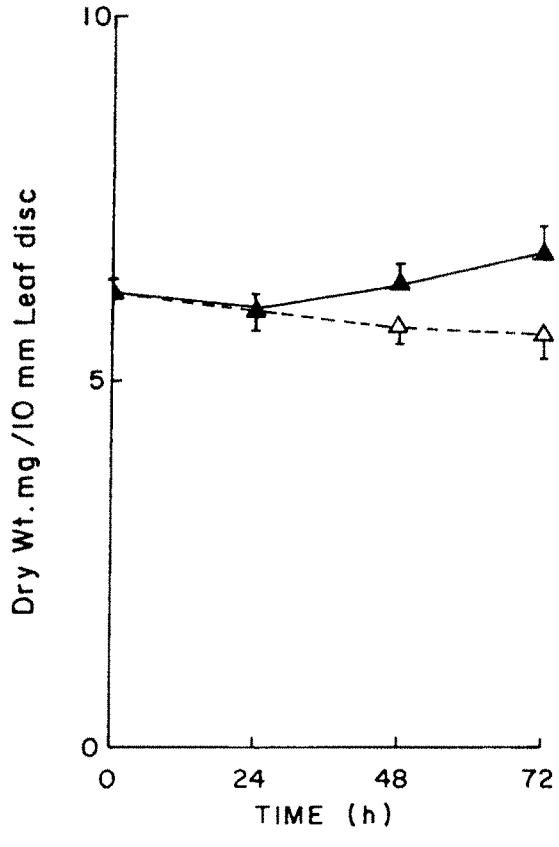
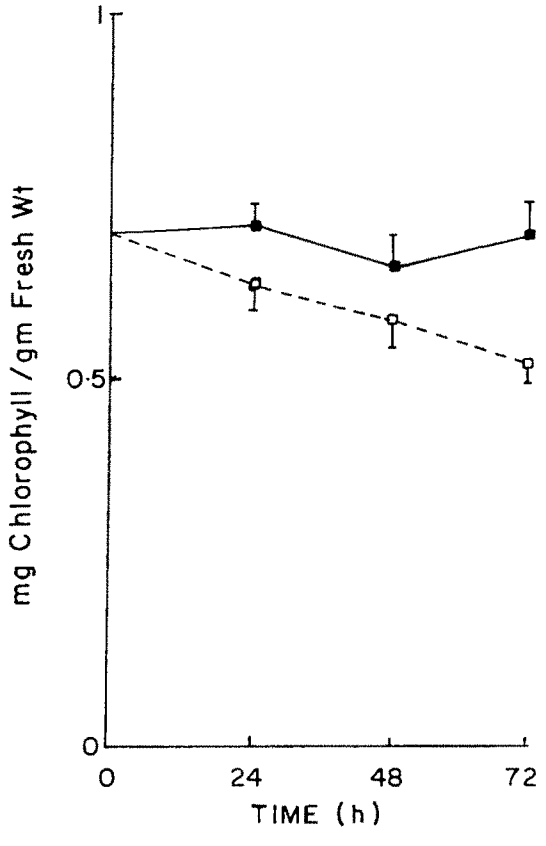
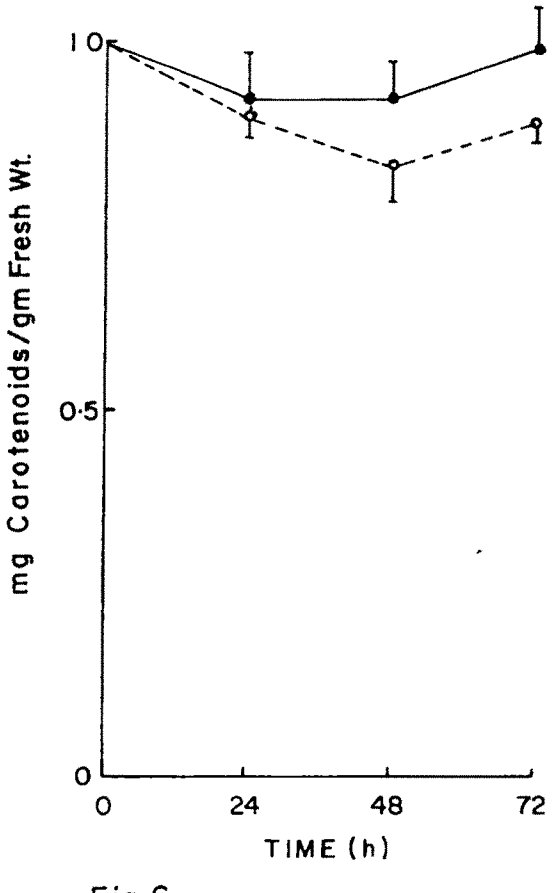


Fig. 5

Fig.6 Effect of 10 μM mevinolin on cultured 10 mm leaf discs of solanum. Dry wt. (a), Fresh wt. (b), Chlorophyll (c) and sterol (d).


Dry Wt. mg /10 mm Leaf disc
10
5
0
0
24
48
72
TIME (h)


mg Chlorophyll /gm Fresh Wt
1
0·5
0
0
24
48
72
TIME (h)


mg Carotenoids/gm Fresh Wt.
1·0
0·5
0
0
24
48
72
TIME (h)

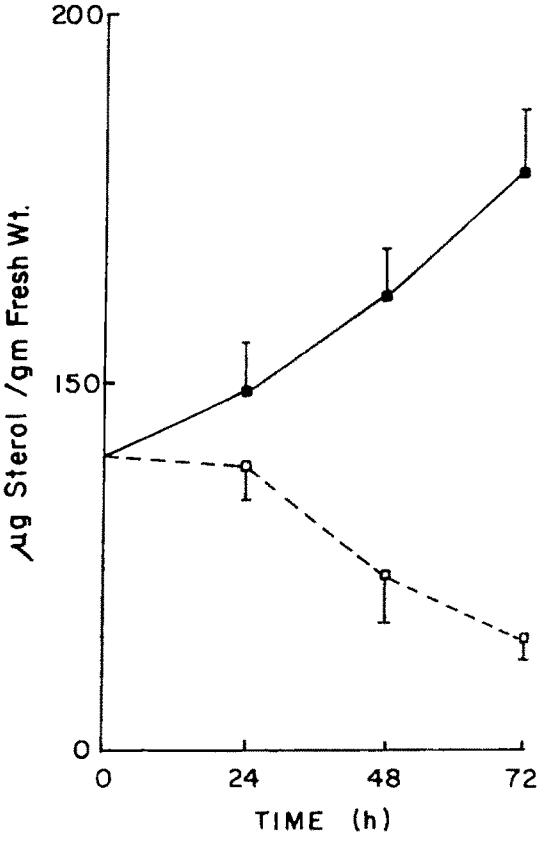

µg Sterol /gm Fresh Wt.
200
150
0
0
24
48
72
TIME (h)

Fig. 6

Fig.7 Effect of mevinolin on cell culture of solanum. Fresh wt. (■——■), Dry wt. (▲——▲) and Solasodine (●——●).

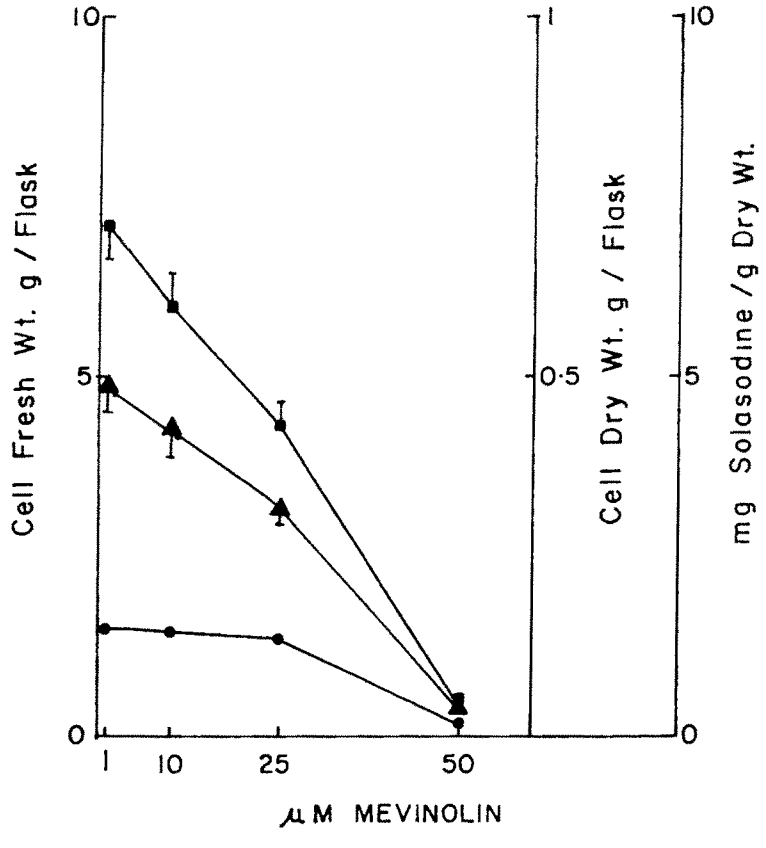

10
5
0
Cell Fresh Wt. g / Flask
1
0·5
0
Cell Dry Wt. g / Flask
10
5
0
mg Solasodine /g Dry Wt.
1
10
25
50
μM MEVINOLIN

Fig. 7

Fig.8 Effect of mevinolin on the sterol content of cell cultures of solanum. Free sterols (▲——▲), steryl esters (●- - -●) and steryl glycosides (■——■).

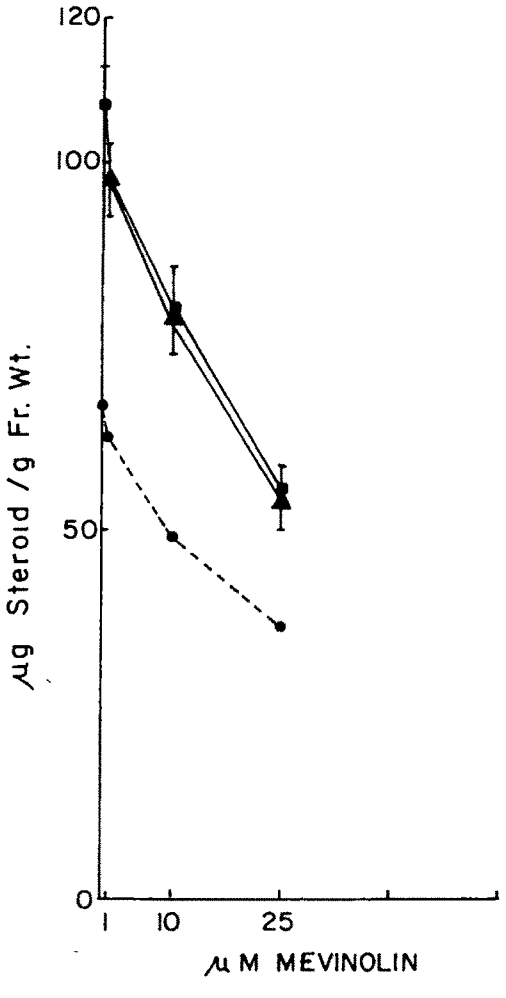


Fig. 8

Fig.9 Effect of mevinolin on plated cells of solanum.

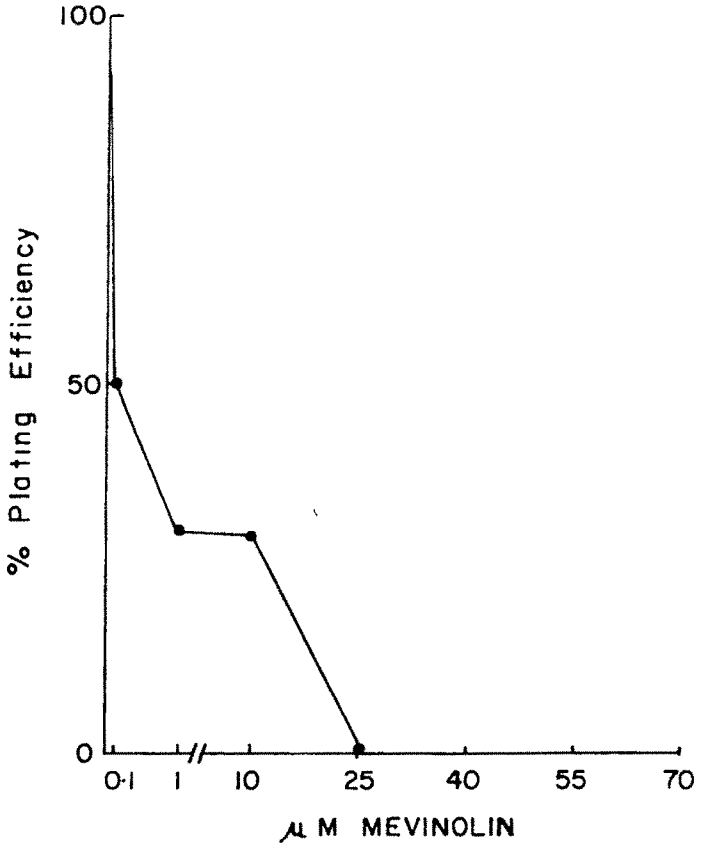


Fig. 9

The plating efficiency decreased in inverse correspondence with the increase in the concentration of mevinolin. Mevinolin at 0.1 µM showed only slight inhibition of the plating efficiency while concentration above 1 µM showed a drastic decrease in the plating efficiency. However, between 1 and 10 µM concentrations of mevinolin a buffering effect was observed in the sense that the plating efficiency showed not much of difference. Conc. of mevinolin at 20 µM was completely lethal as there was no colony formation at all. (Plate 5).

5. Recovery of mevinolin induced inhibition of sterol synthesis and growth by the exogenous supply of various precursors of steroid pathway in different tissue systems:

In order to pinpoint the site of inhibition by mevinolin, various precursors of the steroid pathway such as acetate, mevalonate, squalene and cholesterol were exogenously supplied to the growth medium along with L.D 50 conc. of mevinolin. The reversal effect was determined as a function of growth and sterol accumulation.

1) Reversal with acetate

Acetate being the primary precursor of the lipid metabolism was used at concentrations varying from 100 to 2000 µM in the cultured leaf discs and cell culture systems but it could not bring about any appreciable recovery of the mevinolin induced inhibition (Table 8 & 9 Fig. 10).

Table 8 :Effect of exogenously applied acetate on recovering the 10 μM mevinolin induced growth and sterol biosynthesis in cultured leaf discs of S. xanthocarpum. Data collected after 72 hours of incubations.

Treatment	μg/g fr.wt ± s.e.			Dry.wt.mg/10 mm leaf discs ±S.E
	Free sterol	Steryl ester	Steryl glycoside	
1. Control (=0 mevinolin)	138.83±4.37 a	173.36±4.72 a	187.54±5.37 a	6.28±0.021 a
2. + 10 μM mevinolin	65.96±4.23 b	88.98±3.68 b	91.29±4.41 b	3.09±0.031 b
2.i +100 μM acetate	63.73±3.81 b	95.31±4.31 b	95.46±3.81 b	3.16±0.028 b
2.ii +500 μM acetate	59.78±4.08 b	90.46±4.73 b	88.68±3.68 b	3.00±0.019 b
2.iii +1000 μM acetate	66.82±2.71 b	86.39±3.81 b	90.48±4.31 b	3.21±0.027 b
2.iv +2000 μM acetate	60.63±2.97 b	93.38±4.16 b	86.57±3.32 b	2.99±0.037 b

Values with different letters indicate significant difference at 5% level

Table 9 :Restoration of 25 μM mevinolin induced inhibitory effect on cell cultures of S.xanthocarpum by exogenous acetate.

Treatment	wt.of culture g/flask±S.E		μg/g sterols ±S.E		
	Fr.wt	Dr.wt.	Free sterol	Steryl ester	Steryl glycoside
1) Control (=0 mevinolin)	8.52±5.2 a	0.60±0.03 a	109 ±5.62a	69.6±4.08a	113.4±6.26a
2) +25 μM mevinolin	4.21±4.03b	0.28±0.06 b	50.8+0.08b	35.62±5.32 b	59.32±3.02b
2.i + 100 μM acetate	4.31±5.32b	0.30±0.04 b	52.4±6.12b	34.28±3.38 b	61.81±4.32b
2.ii +500 μM acetate	4.28±4.28b	0.28±0.08 b	53.74±4.82b	38.56±4.72 b	56.00±5.38b
2.iii +1000 μM acetate	3.98±6.32b	0.28±0.07 b	50.00±3.76b	36.00±4.66 b	54.00±2.64b
2.iv +2000 μM acetate	3.86±3.02b	0.28±0.04 b	48.00±7.36b	33.9 ±5.33 b	52.8 ±4.56b

Values with different letters indicate significant difference at 5% level

Fig.10 Reversal of the 10 μM mevinolin induced inhibition of growth and sterol biosynthesis by exogenously supplied acetate. Dry wt (△——△), Chlorophyll (■——■), free sterols (●——●), steryl esters (○--○), and steryl glycosides (▲——▲).

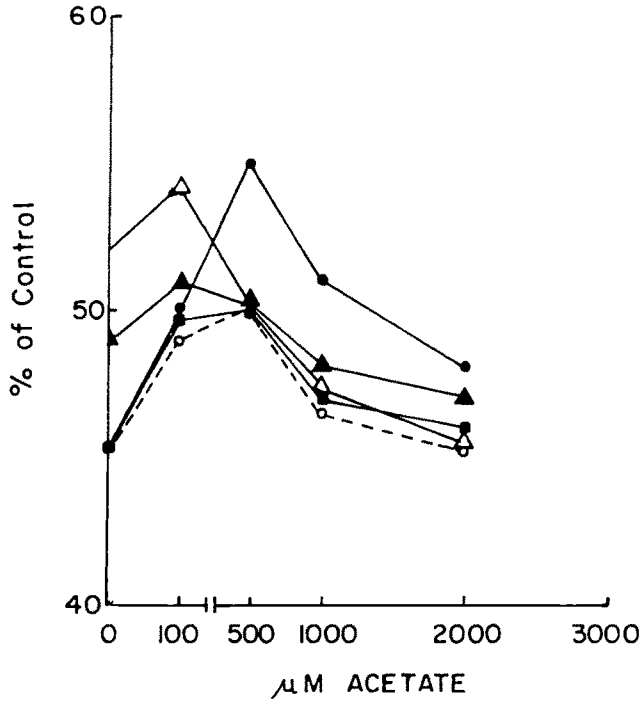


Fig. 10

Reversal with mevalonate

Mevalonate is the first committed precursor of isoprenoid biosynthesis. Therefore mevalonate was exogenously supplied at concentrations varying from 100 to 5000 µM on seedlings, cultured leaf discs and cell cultures to study the recovery of mevinolin induced inhibition by its exogenous application.

It was observed that there was significant recovery in the growth and sterol biosynthesis when mevalonate was supplied in the range of 500 - 2000 µM concentrations, higher concentration of which had adverse effect (Table 10, & 11, Fig.11, 12 & 13).

Reversal with squalene

Squalene is one of the intermediates in the isoprenoid pathway and acts as the direct precursor of cycloartenol in the plant system. Considering the severe inhibition and growth caused by mevinolin, squalene was exogenously supplied to various tissue systems at concentrations varying from 0.1 to 2.5 µM. Concentrations of squalene in this range showed reversal of the mevinolin induced growth inhibition and sterol biosynthesis (Table 12 & 13 & Fig. 14 & 15).

Table 10 :Restoration of10 μM mevinolin induced inhibitory effect on cultured leaf 10 mm leafdiscs of S.xanthocarpum by exogenously supplied mevalonate.

Treatment	Dry.wt.mg/10 mm leaf discs ±S.E	Free sterol μM/gm fr.wt. ±S.E	Steryl ester μg/g fr.wt. ±S.E	Steryl glycoside μg/g fr.wt.±S.E
Control (=0 mevinolin)	7.01±0.02 a	158.00±4.36 a	212.00±4.38 a	203.85±6.32 a
2) +10 μM mevinolin	3.73±0.03 b	83.47±3.08 b	117.68±3.83 b	111.30±4.18 b
2.i 500 μM mevalonate	3.84±0.02 b	90.85±3.28 b	154.00±4.61 b	144.88±3.38 c
2.ii +1000 μM mevalonate	4.27±0.06 b	116.82±3.81 c	169.00±5.31 c	167.4 ±5.02 c
2.iii +1500 μM mevalonate	4.96±0.04 c	118.62±4.84 c	170.6 ±6.43 c	176.85±4.28 c
2.iv + 2000 μM mevalonate	5.02±0.06 d	127.43±3.88 d	169.28±5.31 c	180.67±5.38 d
2.v + 4000 μM mevalonate	4.38±0.03 c	93.00±2.83 b	161.64±4.16 c	158.43±4.71 c
2.vi + 5000 μM mevalonate	3.80±0.03 b	86.36±2.38 b	146.00±3.56 b	136.28±3.05 b

Values with different letters indicate significant difference at 5% level

Table 11: Restoration of 25 μM mevinolin induced inhibitory effect on cell cultures of S. xanthocarpum by exogenously supplied mevalonate

Treatment	g. Wt./flask±S.E		Sterol content μg/g. fr.wt.±S.E		
	Fr.wt.	Dr.wt.	Free sterol	Steryl ester	Steryl glycoside
1) Control =0 mevinolin	8.30±0.230 a	0.53±0.023 a	112.60±5.17 a	67.00±2.30 a	117.6 ±2.90 a
2) 25 μM mevinolin	4.35±0.25 b	0.32±0.012 b	81.3 ±2.57 b	38.00±3.60 b	63.6 ±3.52 b
2.i +1 mM mevalonate	5.1 ±0.11 b	0.33±0.016 b	89.33±3.75 c	47.33±2.33 c	96.00±2.51 c
2.ii +2 mM mevalonate	5.65±0.14 c	0.37±0.008 b	95.00±4.35 c	47.66±3.52 c	98.00±5.17 c
2.iii +3mM mevalonate	6.7 ±0.23 d	0.43±0.023 c	99.66±3.48 d	54.00±2.51 d	106.3 ±4.05 d
2.iv +4 mM mevalonate	6.95±0.31 d	0.46±0.037 c	102.00±4.00 d	58.00±4.04 d	105.35±5.04 d
2.v +5 mM mevalonate	4.95±0.08 b	0.35±0.,016 b	77.00±3.78 c	42.33±4.05 c	92.00±3.21 c

Values with different letters indicate significant difference at 5% level

Table 12 : Restoration of 10 μM mevinolin induced inhibitory effect on cultured 10 mm leaf discs of S. xanthocarpum by exogenously supplied squalene

Treatment		Dry.wt./10 mm leaf discs. ±S.E	Free sterol μg/g fr.wt ±S.E	Steryl ester μg/g fr.wt. ±S.E	Steryl glycoside μg/gm fr.wt. ±S.E
1.	Control '0' mevinolin	5.98±0.06 a	165.00±7.21a	221.66±4.33a	210.00±6.35a
2.i	10 μM mevinolin	3.23±0.02 b	105.66±3.75b	143.00±3.52b	132.00±3.89b
2.ii	+0.1 μM Squalene	3.38±0.03 b	131.66±3.75c	184.00±5.50c	153.3 ±6.06c
2.iii	+0.25 μM squalene	4.03±0.01 c	154.6 ±3.37d	165.00±4.33c	182.3 ±4.05d
2.iv	+ 0.5 μM squalene	3.88±0.04 b	132.00±5.88c	195.6 ±4.42c	165.33±5.38 c
2.v	+0.75 μM squalene	4.18±0.07 c	165.00±5.48d	195.00±4.61c	184.6 ±6.11 d
2.vi	+1 μM squalene	4.99±0.05 c	166.00±5.23d	210.00±4.33d	195.00±4.84 d

Values with different letters indicate significant difference at 5% level

Table 13: Restoration of 25 µM mevinolin induced inhibitory effect on cell cultures of S.xanthocarpum by exogenously supplied squalene

Treatment	Wt./flask g± S.E		Sterol content µg/g ± S.E		
	Fr.wt(gms)	Dr.wt.(gms)	Free sterol	Steryl ester	Steryl glycoside
1) Control 'o' mevinolin	7.20±0.20 a	0.49 ±0.01a	114.66±2.90a	64.00±2.30a	110.00±7.85a
2.i 0.25 µM mevinolin	3.26±0.09 b	0.226±0.01b	56.66±4.66b	32.66±3.71b	67.33±3.71b
2.ii 0.5 µM squalene	4.00±0.06 c	0.267±0.009b	84.66±0.66c	46.00±6.00c	88.00±4.66c
2.iii 1 µM squalene	4.42±0.21 c	0.304±0.019c	90.00±5.03c	46.66±4.05c	90.66±1.76c
2.iv 1.5 µM squalene	5.19±0.11 d	0.353±0.03 d	97.33±6.35d	57.33±4.05d	100.6 ±4.80d
2.v 2 µM squalene	3.66±0.07 b	0.255±0.008c	84.00±3.05c	54.66±1.76d	75.33±2.40c
2.vi 2.5 µM squalene	3.47±0.10 b	0.208±0.01 b	67.33±6.35b	48.66±3.71c	74.58±3.63b

Values with different letters indicate significant difference at 5% level

Fig.11 Reversal of mevinolin induced inhibition of growth and sterol biosynthesis by seedlings of Solanum by exogenously supplied mevalonate.

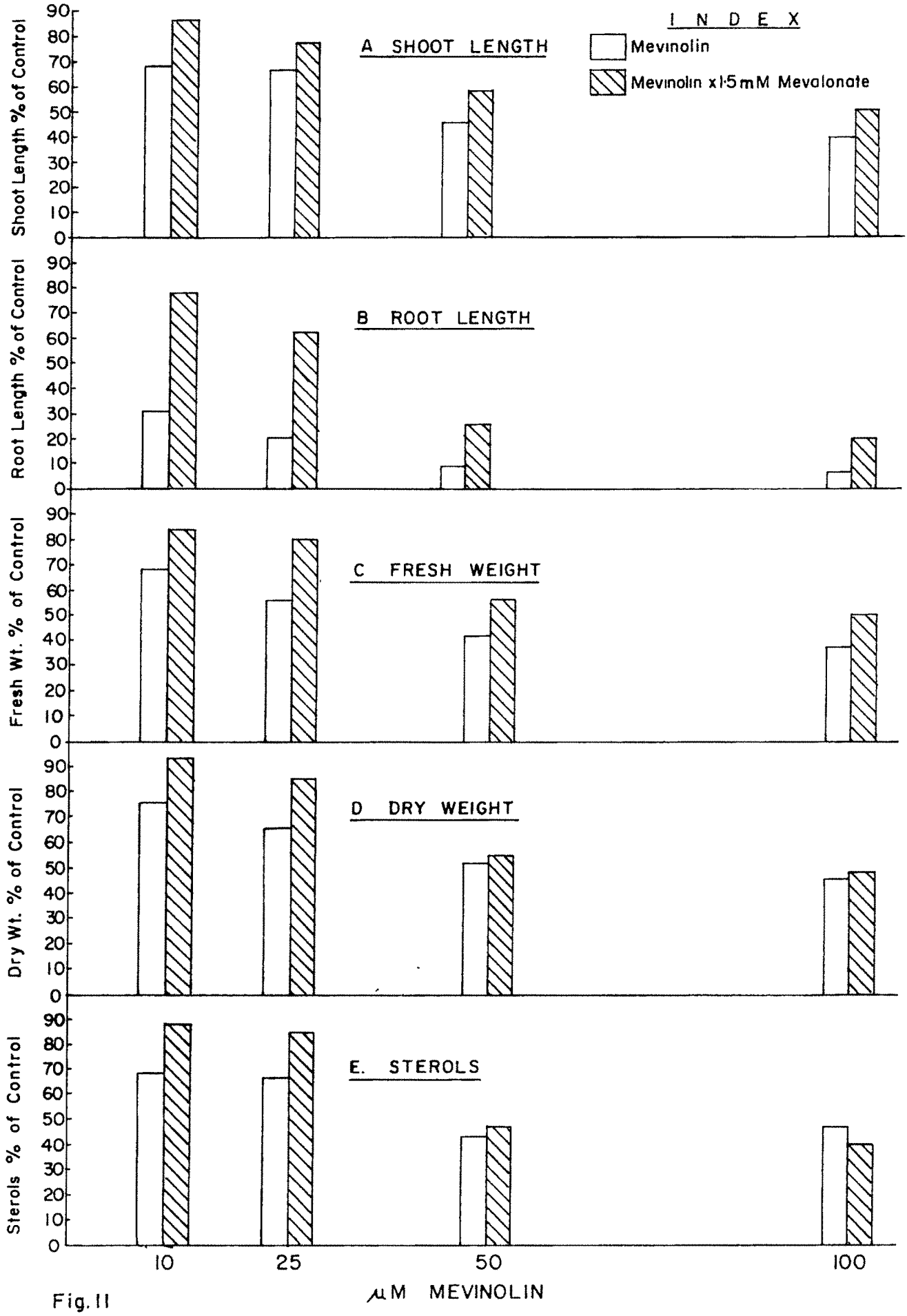

A SHOOT LENGTH
B ROOT LENGTH
C FRESH WEIGHT
D DRY WEIGHT
E. STEROLS
INDEX
Mevinolin
Mevinolin x1·5mM Mevalonate
Shoot Length % of Control
Root Length % of Control
Fresh Wt. % of Control
Dry Wt. % of Control
Sterols % of Control
0
10
20
30
40
50
60
70
80
90
10
25
50
100
μM MEVINOLIN

Fig. 11

Fig. 12 Reversal of 10 μM mevinolin induced inhibition of growth and sterol biosynthesis of cultured 10 mm leaf discs of Solanum by exogenously supplied mevalonate. Dry wt. (□—□), free sterol (●—●), steryl ester (▲—▲) and steryl glycoside (○---○).

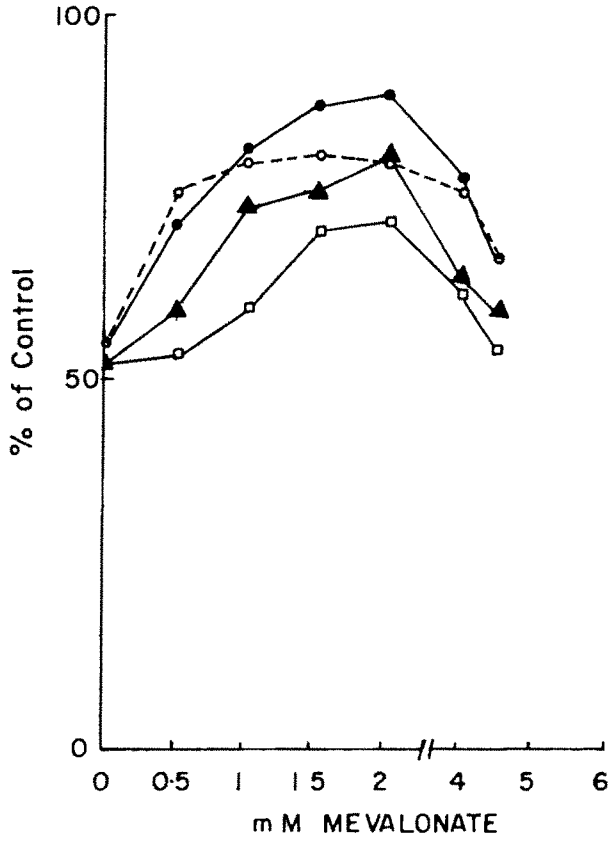


Fig. 12

Fig. 13 Reversal of 25 μM mevinolin induced inhibition of growth and sterol biosynthesis of cell cultures of Solanum by exogenously supplied mevalonate. Fresh wt.(Δ---Δ), dry wt. (o---o), free sterol (o——o), steryl ester (▲——▲) and steryl glycoside (●——●).

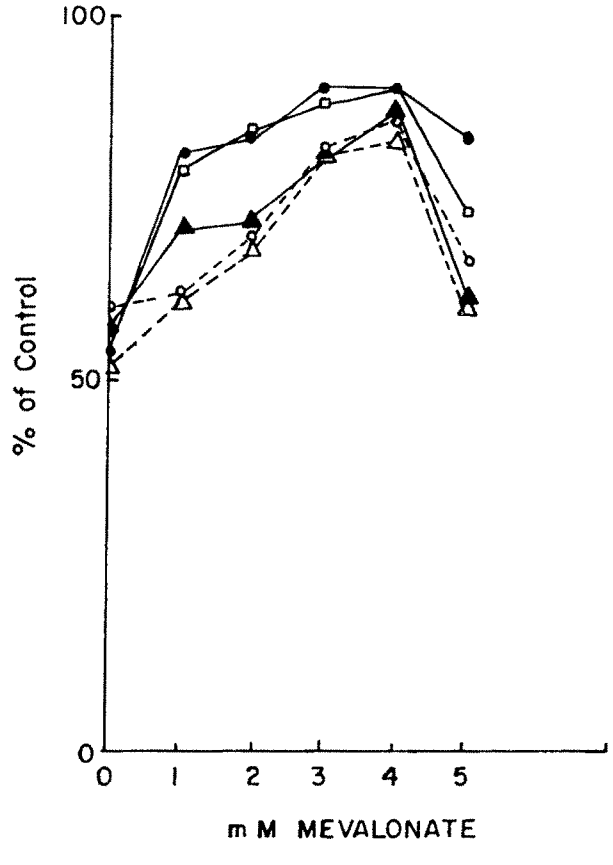


Fig.13

Fig.14 Reversal of 10 μM mevinolin induced inhibition of growth and sterol biosynthesis of leaf discs of Solanum by exogenously supplied squalene. Dry wt. (o—o), free sterol (▲—▲), steryl ester (o---o) and steryl glycoside (•—•).

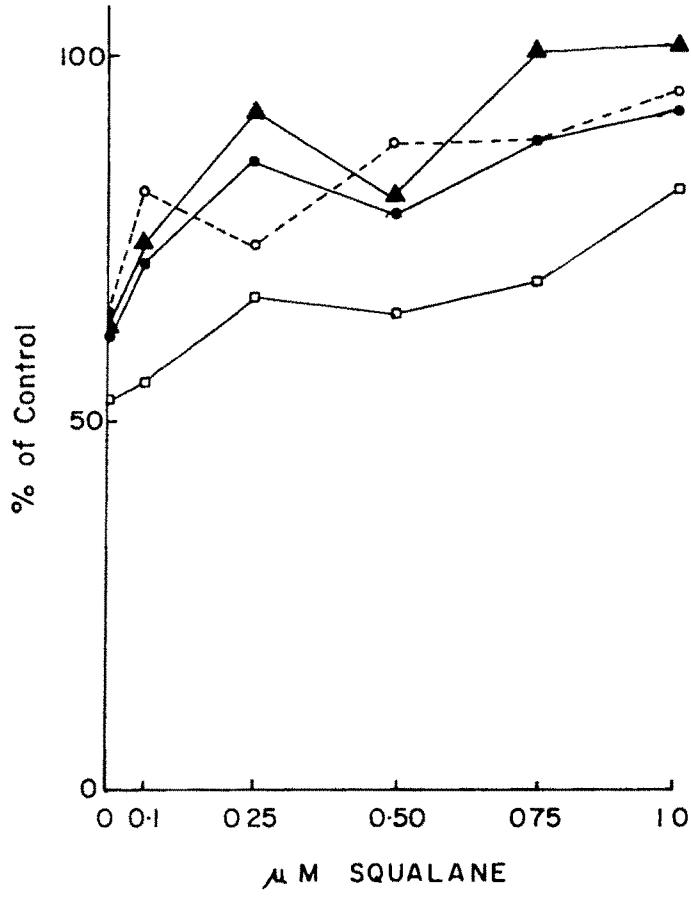


Fig. 14

Fig.15 Reversal of 25 μM mevinolin induced inhibition of growth and sterol biosynthesis of cell cultures by exogenously supplied squalene. Fresh wt. (■——■), Dry wt. (□---□), free sterols (•——•), steryl ester (•----•) amd steryl glycoside (▲——▲).

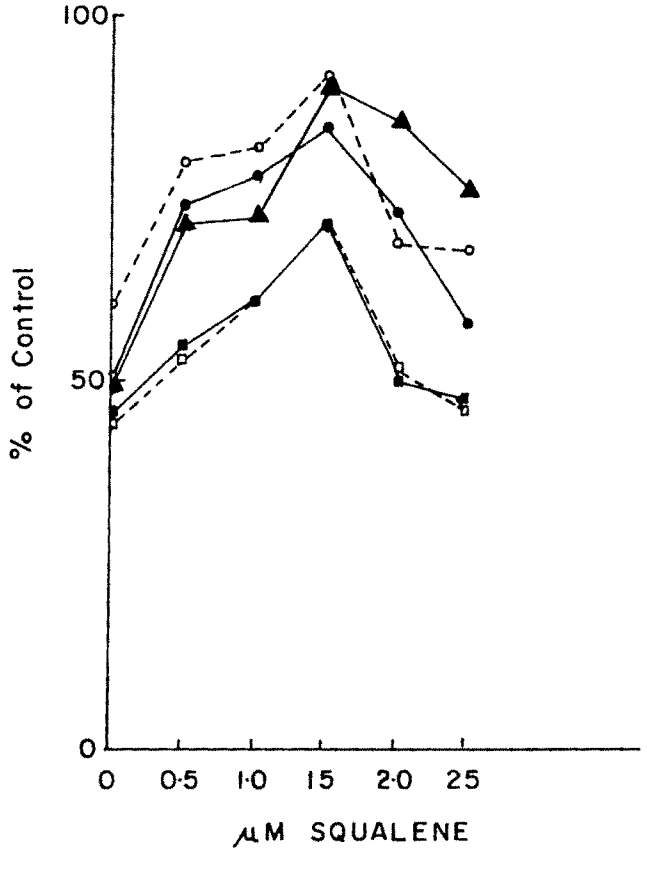


Fig 15

Reversal with Cholesterol

Sterols are shown to be vital molecules with multiple roles in the growth and survival of the plants. Based on this information and considering the inhibition of sterol biosynthesis caused by mevinolin, cholesterol was exogenously supplied to the culture medium to study the reversal of growth inhibition and sterol biosynthesis caused by mevinolin. It was observed that cholesterol can negate the growth and sterol biosynthetic inhibition caused by mevinolin concentration ranging from 01 to1.5 uM (Table 14 & 15 Fig. 16 & 17).

Characterization of the selected cell lines resistant to LD 50 conc. of mevinolin:

Cell lines which were able to grow in presence of L.D.50 concentration (10 μM) of the mevinolin were selected using the cell plating technique as detailed in chapter 2. Cell lines thus selected were grown in the liquid medium (MSL: Table 2.2) for 2 to 3 months with repeated subculture at 9 day intervals. Characterization of this cell line was carried out by studying parameters like growth kinetics in terms of fresh and dry weight increase in the sterol and steroidal alkaloid contents and activity of the gate way enzyme of isoprenoid pathway, HMG COA reductase rate of sterol biosynthesis using ^{14}C acetate.

Table 14 : Restoration of 10 μM mevinolin induced inhibitory effect on cultured 10 mm leaf discs of S. xanthocarpum by exogenously supplied cholesterol

Treatment		Dry. wt./10 mm leaf discs mg ±S.E	Free sterol μg/gm fr.wt. ±S.E	Steryl ester μg/gm fr.wt. ±S.E	Steryl glycoside μg/gm fr.wt ±S.E
1.	Control (0 mevinolin)	6.13±0.071 a	143.00±7.93 a	174.60±7.51 a	184.00±5.29 a
2.i	10 mm mevinolin	3.58±0.042 b	63.00±6.08 b	88.00±6.35 b	96.00±5.19 b
2.ii	+ .1 μM Cholesterol	3.88±0.033 b	113.00±7.51 c	155.6 ±9.52 c	155.66±4.33 d
2.iii	+ .25 μM Cholesterol	4.28±0.046 c	96.2 ±8.60 c	156.4 ±4.63 c	160.66±3.48 c
2.iv	+ .50 μM Cholesterol	4.32±0.038 c	104.5 ±10.97 c	160.00±6.92 c	145.33±5.81 d
2.v	+ .75 μM Cholesterol	4.87±0.026 d	130.33±5.48 e	147.00±9.63 c	145.00±2.08 d
2.vi	+ 1 μM Cholesterol	4.96±0.061 d	132.66±2.02 e	167.6 ±6.64 d	168.00±5.19 c

Values with different letters indicate significant difference at 5% level

Table 15: Restoration of 25 µM mevinolin induced inhibition on cell cultures of *S. xanthocarpum* by exogenously applied cholesterol

Treatment	Wt./flask in gms±S.E		Sterol content µg/gm fr.wt.±S.E		
	Fr.wt.	Dry. wt.	Free sterol	steryl ester	Steryl glycoside
1) Control	7.46±0.32 a	0.53±0.023a	114.00±2.08a	67.00±2.12 a	105.5 ±2.66 a
2) 0.25µM mevinolin	3.83±0.13 b	0.26±0.004b	51.75±2.42b	31.00±1.78 b	40.75±2.68 b
2.i 0.5µM cholesterol	4.7 ±0.29 b	0.33±0.012b	74.75±3.49b	42.75±1.75 c	64.75±2.95 c
2.ii 1 µM cholesterol	5.10±0.12 c	0.34±0.008c	77.5 ±4.05b	49.75±3.44 c	69.5 ±4.05 c
2.iii1.5µm Cholesterol	5.14±0.15 c	0.36±0.01 c	86.5 ±4.25c	57.5 ±3.59 d	78.00±4.02 d
2.iv 2 µM Cholesterol	4.09±0.08 b	0.29±0.004b	69 ±2.27b	37.5 ±2.72 b	67.37±4.67 c

Values with different letters indicate significant difference at 5% level

Fig.16 Reversal of 10 μM mevinolin induced inhibition of growth and sterol biosynthesis of cultured 10 mm leaf discs of Solanum by exogenously supplied Cholesterol. Dry wt. (o—o), free sterol (•—•), steryl ester (o---o) and steryl glycoside (▲—▲).

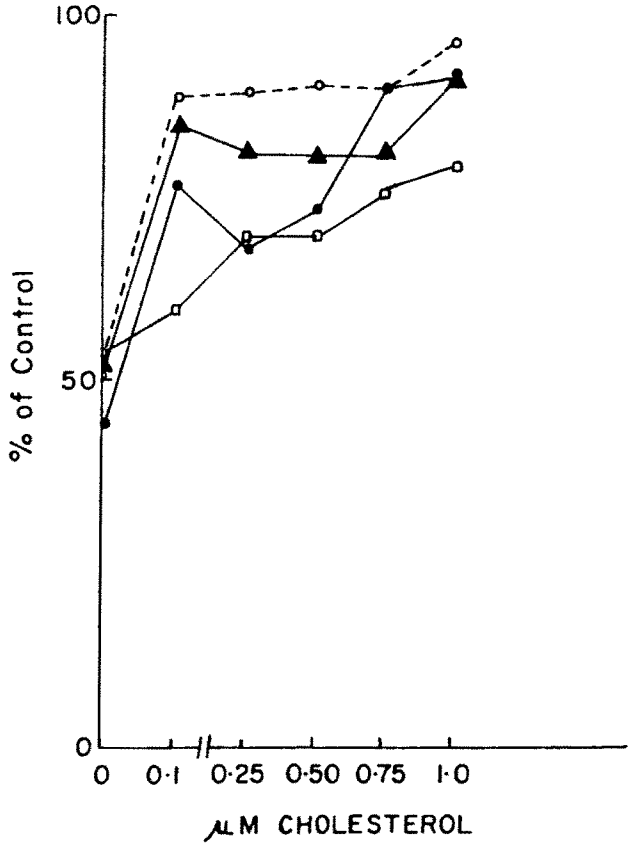


Fig. 16

Fig.17 Reversal of 25 μM mevinolin induced inhibition of growth and sterol biosynthesis of cell cultures of Solanum by exogenously supplied Cholesterol. Fr.wt. (■——■), Dry wt. (□----□), free sterols (●——●), steryl ester (○---○) and steryl glycoside.(▲——▲)

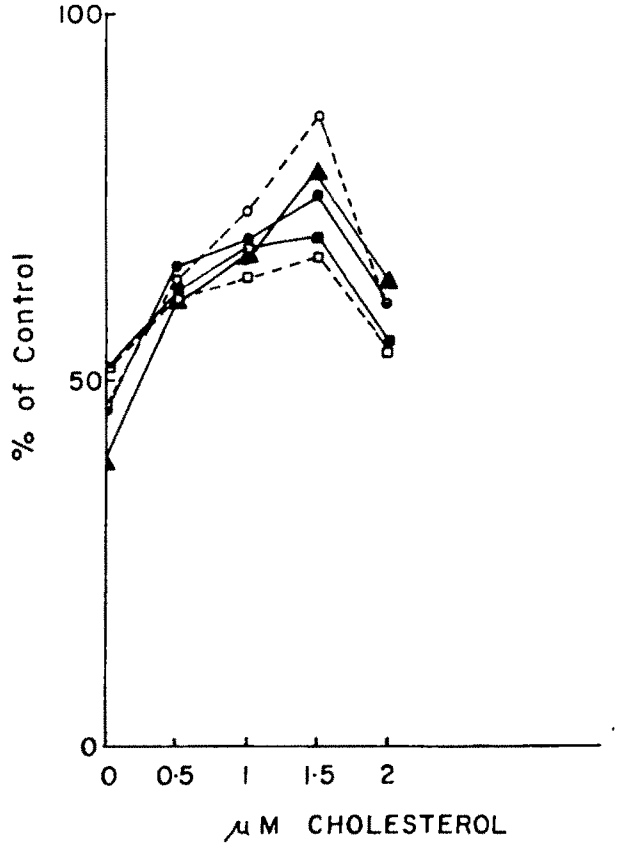


Fig. 17

a. Growth kinetics of the selected cell line:

The selected cell line was grown in the presence of L.D 50 concentration of mevinolin (25 μM) in a single batch cycle. Wild type cell line after incubating similarly in LD 50 conc. of mevinolin was used as control. As is evident from the fig.18.A the growth of resistant cell line in comparison to the wild type cell line was 2 fold more in terms of fresh weight and dry weight though the pattern of the growth remained sigmoid as usual.

When the selected and wild type cell lines were grown under non inhibitory conditions (in the absence of the inhibitor,mevinolin) the fresh weight and dry weight increase did not show any significant difference (Fig. 18.B).

In order to study the changes in the contents of sterol fractions namely free sterols, steryl glycosides and steryl esters similar batch cultures were set up with 6 replicates each. As shown in the figure (19.A) the contents of the free sterols, steryl esters and steryl glycosides of selected cell line remained 1.95, 1.75 and 1.7 fold more than the wild type cell line. On the other hand when both the cell lines were grown in the presence of L.D.50 concentration of mevinolin the contents of sterol fractions declined in the wild type cell in course of time where as the contents of

Fig.18 Comparative growth kinetics of mevinolin resistant and wild type cell lines of solanum. Dotted line indicated wild type cell line.

A. Growth in - mevinolin medium. Fr. wt. (■——■) and dry wt. (o---o).

B. Growth in + mevinolin medium. Fr.wt.(□——□) and Dry wt. (□---□).

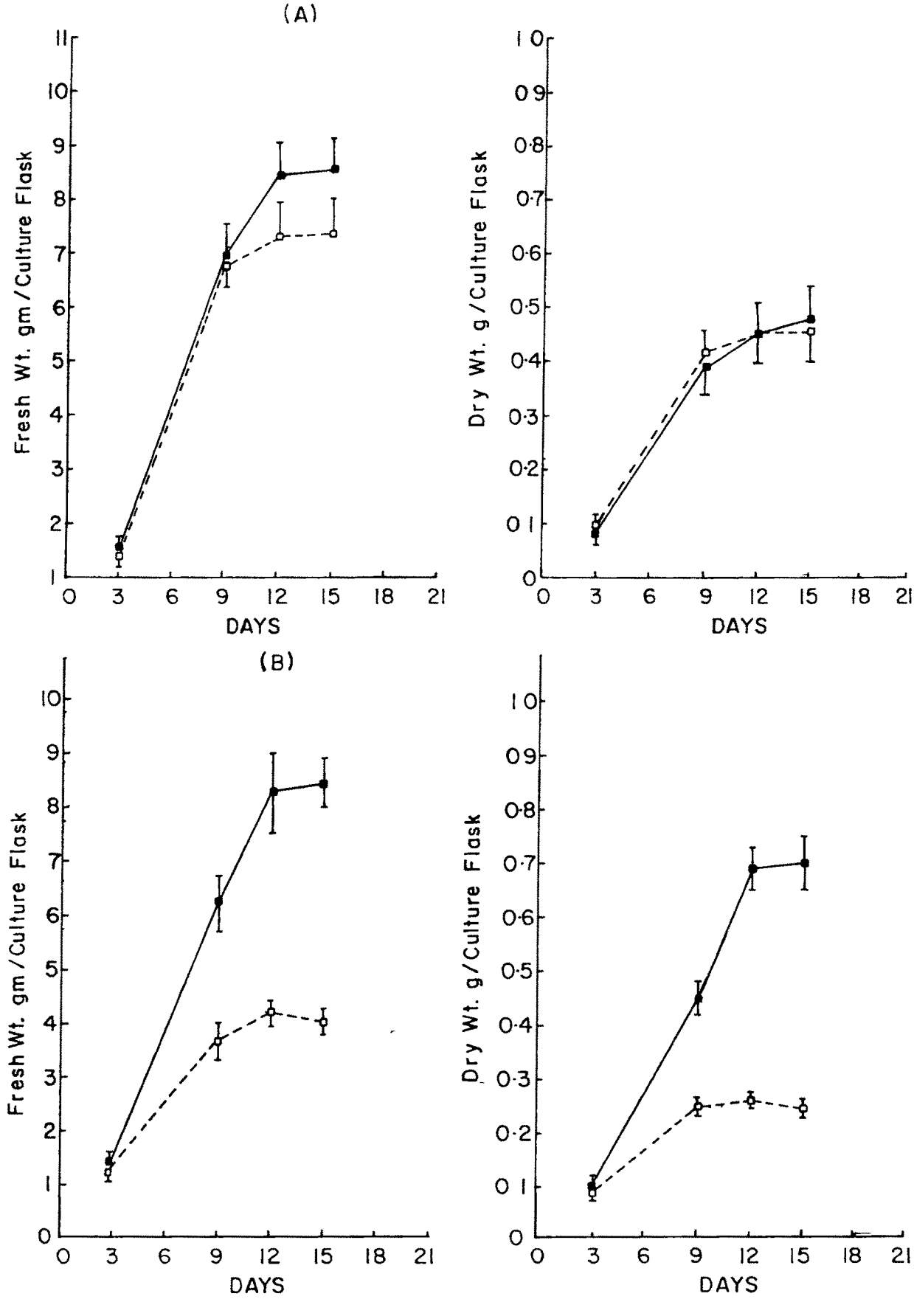

(A)
Fresh Wt. gm / Culture Flask
Dry Wt. g / Culture Flask
DAYS
(B)
Fresh Wt. gm / Culture Flask
Dry Wt. g / Culture Flask
DAYS

Fig 18

Fig.19 Comparative levels of sterols in selected and wild type cell lines of Solanum grown in - mevinolin (A) and + mevinolin (B) media. Dotted line indicate wild type cell line. Free sterol (o—o), Steryl ester (▲—▲), steryl glycoside (□—□).

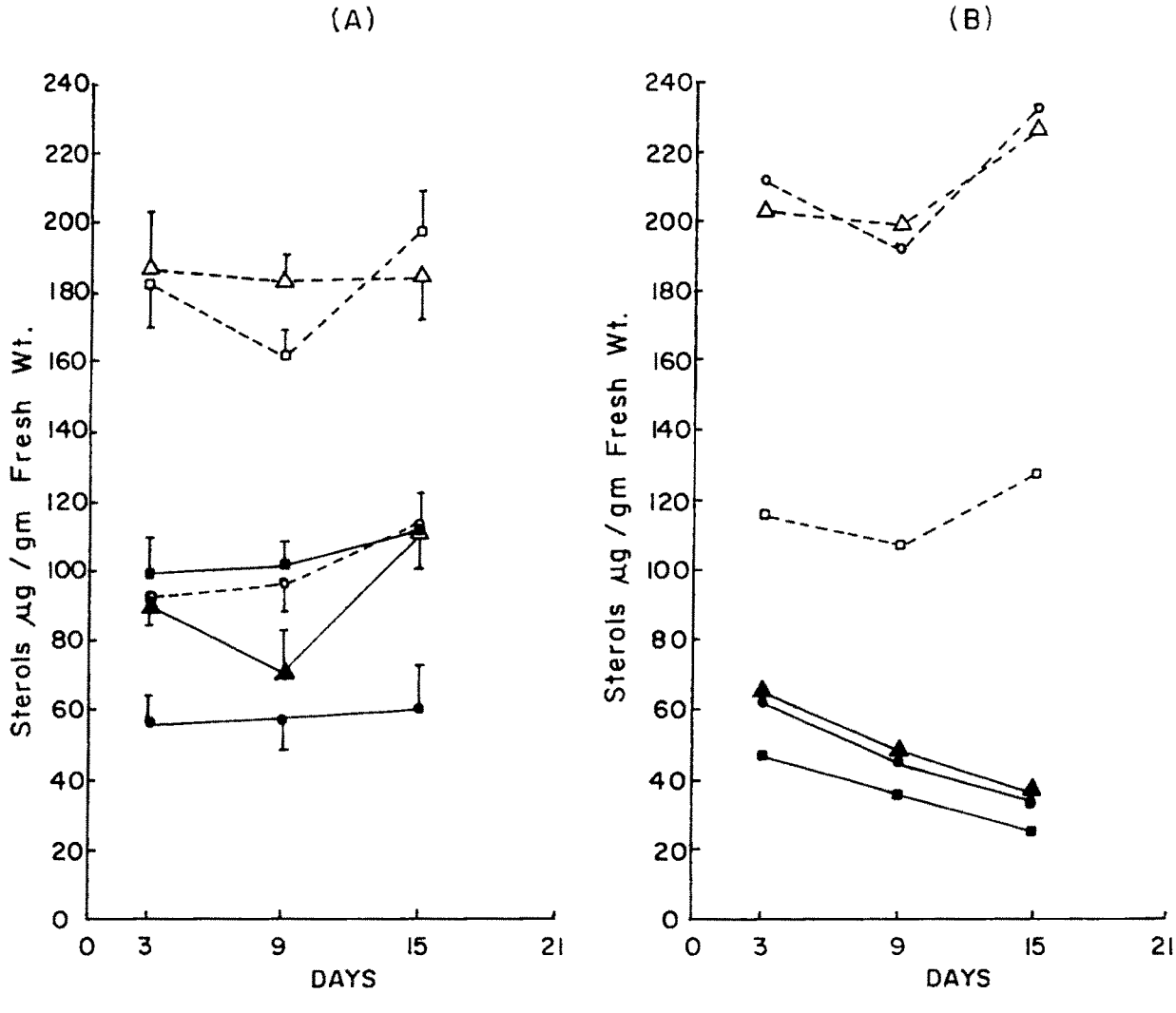

(A)
(B)
Sterols µg / gm Fresh Wt.
240
220
200
180
160
140
120
100
80
60
40
20
0
0
3
9
15
21
DAYS

Fig. 19

sterols fractions viz, free sterols, steryl esters and steryl glycosides showed a similar pattern, remained high on 3rd day, declined on 9th day and again increased to the maximum by 15 day in the selected cell line.

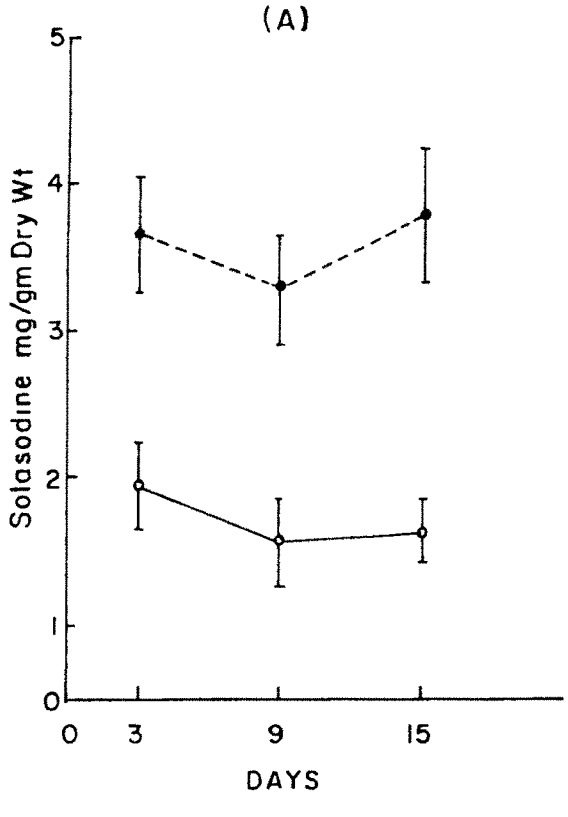
It is interesting to note here that the selected cell line when grown in the presence of mevinolin accumulates greater quantity of steroids rather than when grown in the absence of mevinolin (Fig. 19.B).

Changes in the alkaloid (Solasodine) content of selected cell line:

Since steroidal alkaloid solasodine is derived from the precursor sterols, we were interested to compare its contents in the resistant cell lines with the wild type unselected line. For this both wild type and resistant cell lines were grown in absence and presence of L.D.50 conc. of mevinolin.

The results indicated same pattern of changes in the alkaloid content through the growth cycle but the content of the alkaloid remained 2,2 and 2.2 fold more than that of wild type cell line on 3rd, 9th and 15th day respectively when grown in the absence of mevinolin (Fig. 20.A). When wild type and selected cell lines were grown in the presence

Fig.20 Comparative level of Solasodine in selected and wild type cell lines of Solanum grown in-mevinolin (A) and + mevinolin media. Dotted line indicate selected cell line.


(A)
Solasodine mg/gm Dry Wt
0
1
2
3
4
5
0
3
9
15
DAYS

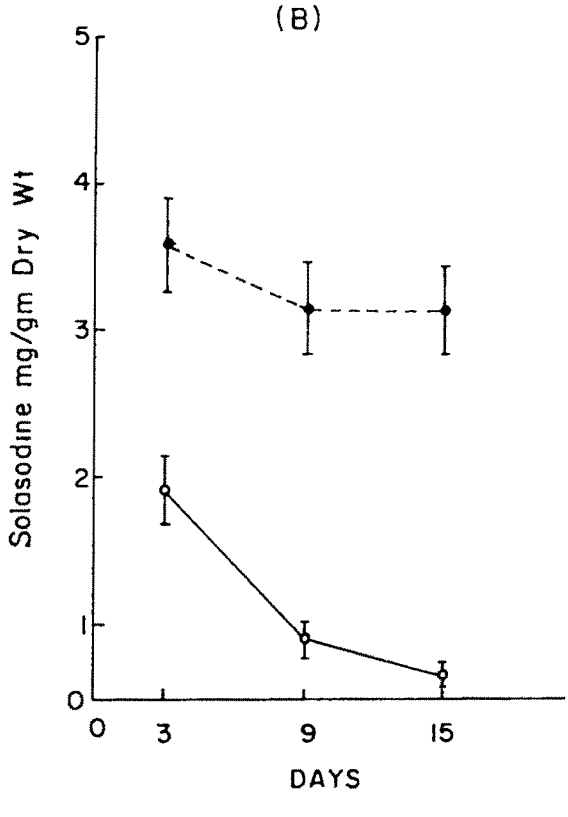

(B)
Solasodine mg/gm Dry Wt
0
1
2
3
4
5
0
3
9
15
DAYS

Fig 20

of L.D.50 concentration of mevinolin the steroidal alkaloid contents were 2, 4.06 and 7.95 fold more in the resistant cell lines than that of the wild type cell line on 3rd, 9th and 15th day respectively (Fig.20.B).

HMG R activity of the cell lines of S.xanthocarpum

As detailed earlier one of the possibilities in developing resistance to the inhibitor mevinolin may be an augmented activity of HMG R enzyme which is inhibited by mevinolin. In order to check this possibility the activity of HMG R in the wild type and selected cell lines were determined.

As the first step to compare the activity of HMG R in the cell lines, effect of pH on the enzyme activity was studied using 3 day old cell cultures of wild type cell line. The optimal pH was found to be 7. (Fig.21). Further, the activity of enzyme HMG R was determined in both wild type and selected cell line at pH 7. As shown in the (Fig.22) the enzyme activity of the selected cell line was 2.5 fold more than that of the wild type cell lines when both cell lines were grown in the MSL medium devoid of mevinolin. Addition of 1 n mol of the inhibitor mevinolin to the reaction mixture inhibited the activity of the enzyme to 78.72% in the wild type cell line.

Fig.21 Effect of pH on the activity of HMGCOA reductase in 3 day old cell culture of Solanum.

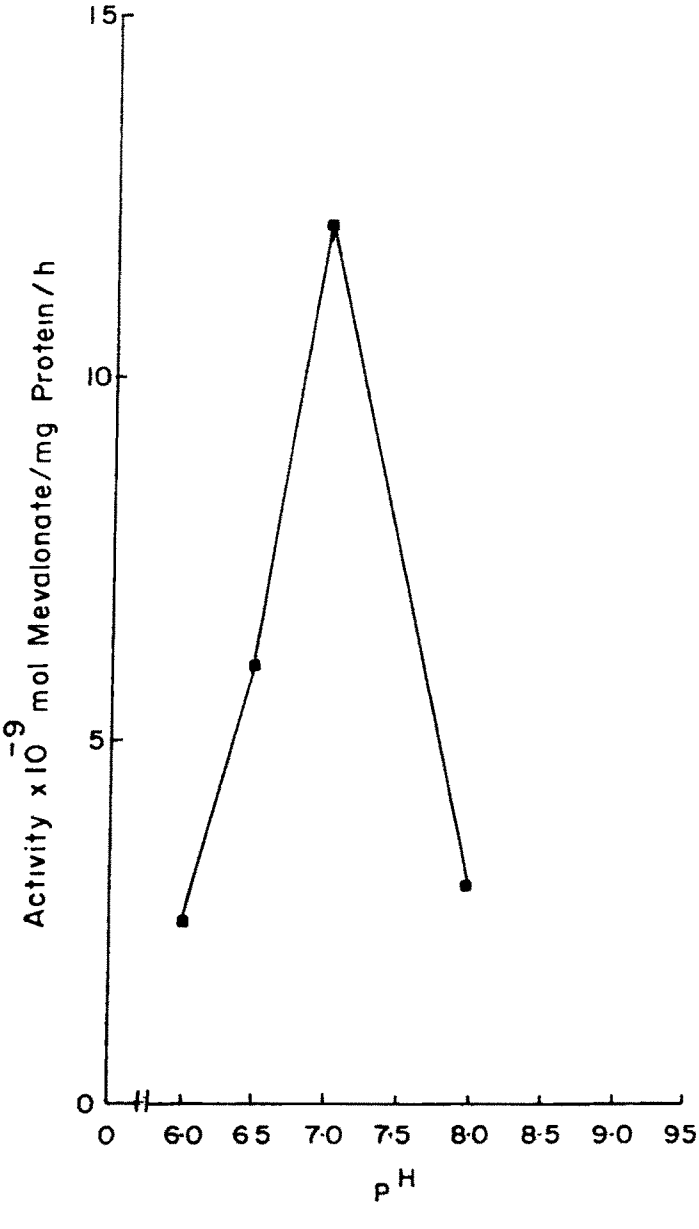

15
10
5
0
Activity x 10^-9 mol Mevalonate/mg Protein/h
0
6·0
6·5
7·0
7·5
8·0
8·5
9·0
95
pH

Fig. 21

Fig.22 Comparative activity of HMGCOA reductase enzyme in wild type (dotted line) and selected 3 day old cell culture of Solanum.

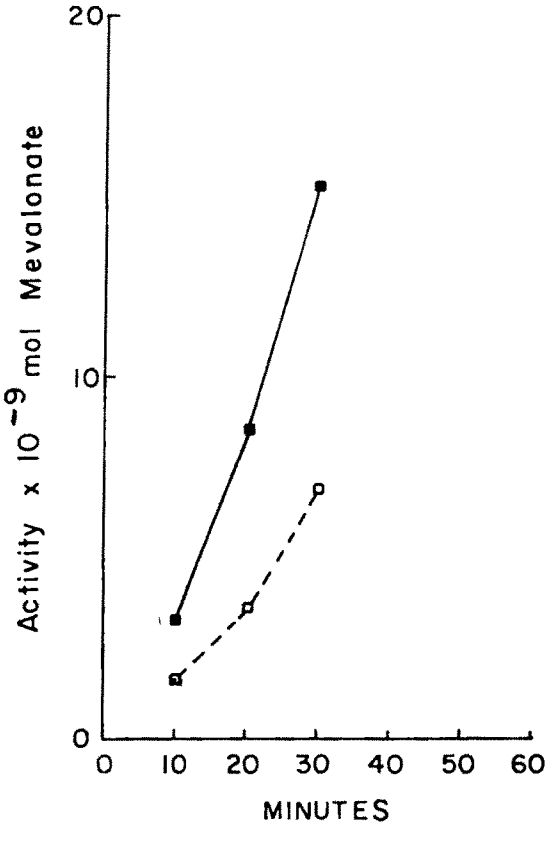

20
10
0
Activity x 10^{-9} mol Mevalonate
0
10
20
30
40
50
60
MINUTES

Fig 22

Comparative rate of sterol biosynthesis in vivo in 3 day old cell cultures of S. xanthocarpum (Wild & mevinolin resistant)

Incorporation studies using 2-^{14}C acetate showed that the rate of incorporation of labelled acetate in to the free sterols, steryl glycosides and steryl esters in the mevinolin resistant cell line was 2.05, 2.38 and 2.23 fold more than the wild type cell line when incubated in the absence of mevinolin for 30 minutes.

Incubation of the cell lines in 10 uM mevinolin supplemented medium showed 35%, 34% and 35% reduction in the incorporation of labelled acetate in to free sterols, steryl glycosides and steryl esters of the wild type cell line while the incorporation of labelled acetate in to sterol, of the mevinolin resistant cell line was not much affected under this condition. Moreover the incorporation of labelled acetate into free sterols, steryl glycosides and steryl esters was 2.86, 2.78 and 3.09 fold more with respect to the wild type cell line (Table 16).

Leaf disc assay of variant regenerants

Leaf discs of 10 mm diameter were cut out from the young leaves of the regenerated plants derived from L.D.50 resistant cell line and were cultured on MS basal medium

Plate 6 Effect of 10 μM mevinolin on cultured leaf discs of S. xanthocarpum.
a) leaf discs from regenerated mevinolin resistant plants treated with mevinolin b) control (0 mevinolin) c) leaf discs from the wild type plants treated with mevinolin.

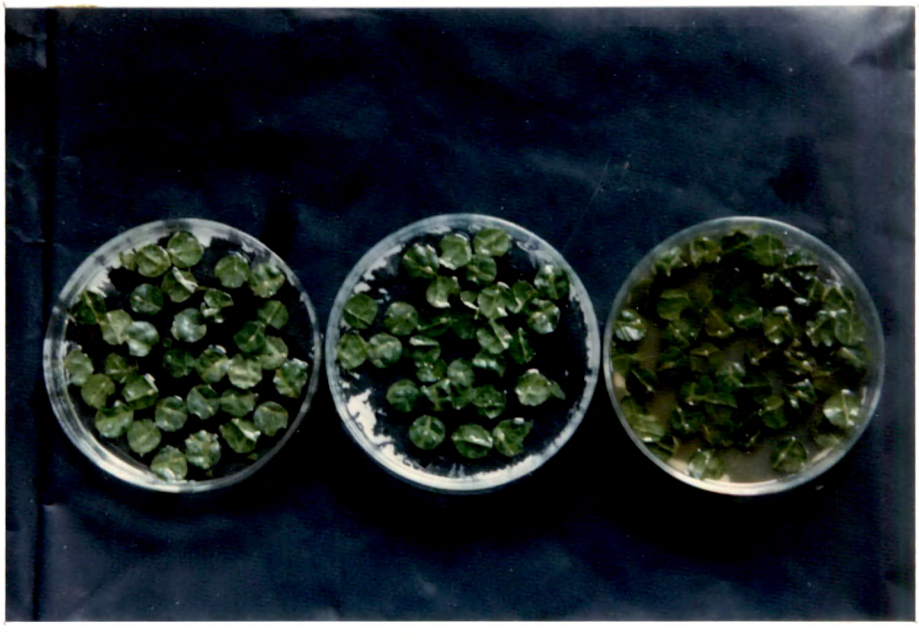


Table 16 : % incorporation of $2^{14}C$ acetate in to steroids in variant and wild type cell cultures of *S. xanthocarpum.*

Time	Wild type cell line						Mevinolin resistant cell line					
	Without mevinolin			With mevinolin			Without mevinolin			With mevinolin		
	Free sterol	Steryl ester	Steryl glyco-side	Free sterol	Steryl ester	Steryl glyco-side	Free sterol	Steryl ester	Steryl glyco-side	Free sterol	Steryl ester	Steryl gluco-side
10'	0.38	0.31	0.18	0.30	0.23	0.14	0.82	0.51	0.32	0.73	0.38	0.32
20'	0.68	0.36	0.21	0.44	0.27	0.11	1.24	0.72	0.29	0.97	0.41	0.34
30'	0.69	0.29	0.17	0.46	0.19	0.11	1.42	0.69	0.38	1.32	0.53	0.34

supplemented with 2% sucrose, 10 μM mevinolin and no hormones. Similarly cultured leaf discs in the absence of mevinolin and leaf discs from wild type plants cultured under similar conditions served as the control. All cultures were incubated at 26 ± 2°C and 16 hour photoperiod. Loss of fresh and dry weight was considered to measure the effect of mevinolin on these cultured leaf discs. Weight determination after 72 hours of incubation showed that plant No.1 and 3 has no apparent loss of fresh weight or dry wt. compared to the control under 10 uM mevinolin stress while plant No,.2 showed significant loss of weight (Fresh &Dry) under this condition. All the wild type leaf discs also showed upto 50% loss of dry and fresh weight in 72 hours of incubation (Table .17).

Analysis of the steroidal contents of variant regenerants

To further characterize these regenerants analaysis of the β-C-3 sterols (Free, esterified and glycosylated) in young leaves was carried out. This analaysis revealed that plant No. 1 and 3 possess 1.25 & 1.23 fold more free sterols, 1.33 and 1.35 fold more steryl ester and 1.30 & 1.35 fold more steryl ester and 1.30 and 1.20 fold more steryl glycosides while plant No.2 showed almost same amount of β C-3 sterols compared to the wild type plants.

Table 17 : Effect of 10 μM mevinolin on the growth of cultured leaf discs of mevinolin resistant S. xanthocarpum. The values are average of 5 independent determinations ± S.E. Leaf discs were cultured in MS basal medium for 72 h under 16 h photoperiod 25 ± 2°C temperature.

Variant	Fresh wt.at '0' time mg/leaf disc ± S.E.	Fresh wt. after 72 hours		Dr.wt.'0' time mg/leaf disc ± S.E	Dry.wt.after 72 hours	
		Control	Treated with mevinolin		Control	Treated with mevinolin
		mg/leaf discs ±S.E			mg/leaf disc ± S.E	
Plant I	32.2±2.92a	37.21±2.58 a	34.38±3.22a	3.32±0.20a	3.5±0.38a	3.46±0.36a
Plant II	30.4±2.07a	31.33±2.42 a	21.31±2.12b	3.16±0.18a	2.9±0.28b	1.94±0.23b
Plant III	33.2±3.11a	32.46±3.38 a	33.6 ±3.38a	3.43±0.23a	3.5±0.62a	3.48±0.52a
Wild type	56.8 ±3.89a	55.40±3.39a	28.56±3.68b	6.22±0.43a	5.6±0.46a	3.38±0.57b

Values with different letters indicate significant difference at 5% level

Analaysis of the solasodine content in the dried young leaves showed upto 3.56 mg/g dry wt. which is 1.73 fold more than that of wild type. However one of the variant plants analysed (Plant.II) showed a bit less (1.89 mg/g dry wt.) Solasodine (Table 18).

Table 18 : Analysis of β-C-3 sterols and solasodine in the selected regenerant S. xanthocarpum. Young leaves of the1½ month old plants were used as the source tissue

Regenerant Plant No.	Free sterol µg/gm fr.wt. ±S.E.	Steryl ester µg/gm fr.wt. ±S.E.	Steryl glycoside µg/gm fr.wt. ±S.E.	Solasodine mg/gm dr.wt. ±S.E.
1	223.00±6.24 a	101.66±4.04 a	190.66±9.01 a	3.36±0.20 a
2	164.66±9.45 b	67.00±9.84 b	133.66±6.65 b	1.89±0.16 b
3	219.00±11.93a	102.00±7.02 a	188.00±10 a	3.56±0.41 a
Control	178.66±7.02 b	76.00±6.24 b	147.33±9.01b	2.06±0.10 b

Values with different letters indicate significant difference at 5% level