D I S C U S S I O N

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DISCUSSION

Growth characteristics of cell suspension cultures of Solanum xanthocarpum:

Growth of cell culture was best on MS medium supplemented with growth regulators (0.5 ppm) 2,4-D, kinetin (0.1 ppm) and casein hydrolysate (250 ppm). The growth curve was typically sigmoid with a lag phase of 6 days, log phase up to 12th day followed by the stationary phase (Fig.1). The pattern of changes in the sterols and steroidal alkaloid (solasodine)showed a zigzag pattern as shown in Fig. 2. These results are similar to the results obtained by Subramani (1991) who studied the growth characteristics of Solanum xanthocarpum under similar conditions.

Cell plating and plating efficiency

Maximal plating efficiency (75%) was achieved when $4x10^4$ cells were plated on 9 cm petridish containing MS medium supplemented with 0.5 ppm 2,4-D, 0.1 ppm Kn & 250 ppm casein hydrolysate. This result again coincides with the observations of Subramani (1991).

Morphogenetic potential of cell cultures of S.xanthocarpum

Regeneration of Solanum xanthocarpum from callus

derived from different explants such as defoliated shoots (Rao and Narayana Swamy, 1968), leaf segments (Prasad and Chaturvedi, 1978) and mesophyll protoplasts (Saxena, et al.,1982) is reported earlier. In our experiments we found that regeneration of S. xanthocarpum from one and a half years old cell cultures is possible at high frequency. High rate of shoot formation (upto 90%) from the single cell derived colonies of 14-16 months old cell cultures was obtained on MS medium supplemented with 10 μM BAP and 0.1 μM IBA. Regeneration frequency (80%) from 18-20 months old cell culture is reported previously (Subramani, 1991). Organogenesis is greatly influenced by the age of the callus cultures (Flick et al, 1983). This may be the reason for the observed 90% regeneration frequency in our experiment when compared to the 80% regeneration by Subramani (1991).

In vitro root formation from shoots was achieved on 3/4 strength MS medium derived from wild type (unselcted/ control) cell lines but combination of auxins (IBA and NAA) were required to induce rooting of the shoots derived from selected mevinolin resistant cell line. Many of the herbaceous members have shown to root without any added auxin (Flick et al., 1983). However, it is evident that all varieties of same species are not equally responsive to the added growth hormones (Krikorian et al, 1987). The observed requirement of auxin for rooting of mevinolin resistant

shoots may be attributed to the augmented isoprenoid synthesis which is involved in the synthesis of natural cytokinins.

Transplantation of the regenerated plants to pots.

Transfer of *in vitro* propagated plants to the *in vivo* conditions requires tedious acclamatization process (Baker, 1974; Sutter & Hanghans; 1979). The observed low frequency of survival can be attributed to the dessication caused by rapid and uncontrolled dehydration. This rapid loss of water is because of the under developed cuticle, stomata and vascular system (Driver, 1986). Poor rooting or anatomical anomaly of the root shoot vascular juncture greatly hampers the water absorption capacity of such plants. When compared to the green house plants *in vitro* regenerated plants have lesser photosynthetic efficiency due to the high concentration of nutrients supplied during *in vitro* culture (Dustan and Turner, 1984). In order to overcome this difficulty, many workers have suggested various techniques with limited success. As described by Anonymous, (1988) use of anti transparents, reduction of ammonium and elimination of cytokinins could not help in improving the rate of survival of the micro-propagated plants. The application of simple technique (Selvapandyan *et al.*,1988) also was of not much use but gradual acclamatization with controlled

reduction in humidity and increase in the light intensity was best solution for acclamatization of these micropropagated plants.

Steroid analyses of the plant organs of Solanum xanthocarpum

As summarised in the (Table 3) young leaves showed maximum free sterol (181.3 ug/g.fr.wt.) steryl glycosides (146.5 ug/gm fr.wt.) but, steryl ester content was maximum in young stem parts while steroidal alkaloid content was more in young berries (3.66 mg/g. dry.wt.) compared to all other plant parts.

Sterol content of Solanum xanthocarpum in the plant organs is not reported elsewhere. Steroidal alkaloid, solasodine in the plant parts is determined by previous workers (Atal and Kapoor; 1976, Weiler et al., 1980). The estimated amount of solasodine is lower than the reported value of solasodine from dried leaf material of Solanum by Atal and Kapoor (1976) and other plant parts viz; stem and roots by Weiler et al., (1980). This observed difference can be due to the effect of edaphic factors as well as the climatic factors in which the plants are grown. Similar variation in the alkaloid content of S. dulcamara was observed from the reported values due to edaphic and climatic factors (Bhatt and Bhatt, 1984a). The difference in

the analytical procedure used by us could also be another reason for the observed variation. The heterogenity of the Solanum plants due to the cross pollinating nature is another factor which has influenced this variation. Chandler and Dodd (1983.a) have also shown that variation in solasodine content is associated with the growth conditions of the plants in question.

Analysis of free sterol, steryl ester and steryl glycosides in batch cell cultures of S. xanthocarpum

From Fig.2 it is clear that the sterol content showed great fluctuation during the growth cycle. The results indicated that free sterol synthesis is not restricted to any particular phase of growth, while steryl glycosides and steryl esters appear to accumulate during stationary phase. Quantum of steroidal alkaloid solasodine also appears to be produced maximally in the stationary phase.

Secondary metabolism in plants is genetically controlled, can be influenced to some extent by environmental factors. The in vitro studies on secondary metabolites have shown that it is influenced by many factors (Marshall and Staba, 1971; Hosoda et al., 1979; Bhatt et al., 1983; Chandler and Dodd, 1983 a,b, 1984; Manek et al., 1984; and Quadri Giuleti, 1990). Factors influencing

biosynthesis and accumulation of steroidal alkaloids in Solanum is studied in detail (Heble et al., 1971; Bhatt et al., 1983, Chandler and Dood, 1983 b). Heble et al (1968) showed the steroidal alkaloid synthesis by S. xanthocarpum. Hosoda and Yatazawa (1979) described the alkaloid production in S. laciniatum in the stationary phase. Existing reports are contrary regarding the growth pahse at which the alkaloid production takes place. Jain and Sahoo (1981) reported the alkaloid production in the growth phase of S. jasminoides while, Chandler and Dood (1983,a) Emke and Eilert (1986) showed that the alkaloid production is not restricted to any particular phase of growth.

Effect of mevinolin on the growth of seedlings, cultured leaf discs and cell cultures of S. xanthocarpum

Results presented in Table 4 and 5 indicate that the growth of roots is inhibited to a greater extent than that of shoots. Further the degree of inhibition is more under dark conditions in comparison to light conditions. Accumulation of sterol was inhibited by more than 50% by 50 to 100 uM mevinolin Fig. 4 & 5 . Reversal of the growth and sterol metabolic inhibition caused by mevinolin was achieved by exogenous supply of mevalonate Fig.11.

Time course study on inhibitory effect of mevinolin on cultured leaf discs indicated that it was seen very clearly

by 72 hours of incubation. Fig.5 & 6,Table 6&7. However, the inhibition of plastidic isoprenoid compounds (Chlorophyll & carotenoids) was not pronounced as compared to its effect on growth and sterol biosynthesis.

Growth, sterol and steroidal alkaloid synthesis of the cell culture were inhibited to more than 50% of control by 25 μM concentration of mevinolin (Fig.7). Free sterol content was reduced to 37% of the control while steryl glycoside and steryl ester contents were reduced to almost 50% of the control (Fig.8).

Sterols and triterpenoids act as architectural and functional components of endoplasmic reticulum and plasmalemma, presumably through specific interaction with fatty acids and proteins (Nes and Mc Keam, 1977; Nes and Heftmann, 1984). Plastoquinones are integral components of plastid and mitochondrial electron transport chain. (Lichtenthaler, 1979, 1980; Rudney 1977). The autotrophy of plants is effected by the help of important isoprenoid plant pigments viz; Chlorophylls and Carotenoids (Davis, 1977; Scheider, 1975). Invariably all the above mentioned important biomolecules in plants are isoprenoid products whose ultimate precursor is mevalonate. In addition to these compounds informational molecules of the plants such as

gibberellins (Greabe, 1978), isopentenyl adenine and abscisic acid (Milborrow, 1974; McGaw, 1987) are also products of this pathway whose precursor is mevalonic acid.

Mevinolin inhibits the enzyme HMGR responsible for mevalonate synthesis (the first precursor of isoprenoid biosynthesis) and subsequently growth of the plants. (Bach and Lichtenthaler, 1982). Bach & Lichtenthaler (1983) showed that there is no effect on germination of seeds of radish even when mevinolin was used at concentration upto 500 µM. Using radish seedling they showed that 10-100 ppb of mevinolin can cause significant reduction in the root growth. When 500 µM of .... mevinolin was used they could achieve 86% reduction in the sterol content of radish seedling. Further they showed that to achieve significant reduction in pigment accumulation, upto 500 uM of mevinolin was required which can reduce the prenyl lipid content to 44% of control in the etiolated thin primary leaf segments of wheat. Bach reported the effect of mevinolin on cell cultures of Sylibum marianum. They observed that 0.625 & 10 µM of mevinolin can reduce the growth (on dry wt. basis) to 85% and 28% of control while free sterol to 76% and 8% of the control respectively. Maurey et al (1986) showed that the growth of unicellular green alga Ochromonas malhamensis is completely inhibited by

200 μM of mevinolin. A time course study using 10 and 100 μM of mevinolin at one hour & 2 hours exposure reduced the sterol content in this alga to 9.3%, 6% and 18% & 14% of control respectively. However, 3 day exposure to 10 μM mevinolin showed an increase to 103% sterols with respect to the control.

Ceccarelli and Lorenzi (1984) using callus cultures of sunflower showed that 2.5×10^{-5} M of mevinolin inhibited the growth completely. They also showed that compactin another inhibitor of HMGR also produces similar effect.Our observations on the inhibitory effect of mevinolin on seedlings, leaf discs and cell culture are similar to the previous observations though the concentrations of the inhibitor used and the extend of inhibition was not the same.

The inhibitory effect of mevinolin on pigments was not pronounced when compared to steroids. This discrepancy "precursor being same (Mevalonate) the synthesis of Chlorophylls and carotenoids were not much affected" can be attributed to the compartmentalization of isoprenoid enzymes in to the plastids whose membranes may not be equally permeable to mevinolin. In a previous study Bach and Lichtenthaler (1983) have shown this type of a differential

inhibition of isoprenoids by mevinolin in wheat leaf segment system. They have interpreted the inability of mevinolin to inhibit the chlorophyll synthesis because of the low transport rate of mevinolin through the plastidmembrane. However, when the concentration of mevinolin was increased to 500 μM even chlorophyll synthesis was inhibited (Bach & Lichtenthaler, 1983). In the present study the mevinolin concentration used was low (10 μM) but after 72 hours of incubation, low inhibition of the chlorophyll accumulation was noticed. The reason for the low inhibition of chlorophyll synthesis can be because I used young leaves which were active in synthesis of prenyl pigments. This observation also coincides with that of previous report (Bach and Lichtenthaler,1983) when leaf segments of wheat were used as the experimental material. Inhibition of growth by mevinolin can be attributed to the inhibition of mevalonate formation and subsequent shut down of higher order isoprenoid synthesis. There are numerous reports on inhibition of plant growth by inhibitors of isoprenoid biosynthesis other than mevinolin (Douglas & Paleg,1974, 1981; Hosokawa et al, 1984; Bach, 1985; Fletcher et al, 1986; Burden et al, 1987; Haughan et al, 1987). Douglas & Paleg (1974) have studied the effect of AMO 1618, CCC, phosphon D & B 995 on the elongation growth of 21 day old seedlings of tobacco. They observed that AMO 1618, CCC,

phosphon D and B 995 at concentrations 3, 10 & 30 µg/plant for 8 days can reduce the shoot length of the seedlings by 8%, 18%, 40%, 4%, 8%, 20%, 39%, 52% and 54% respectively. In a later report (1981) they have studied the effect of SKF 7732-A and AY 9944 at 100 and 300 µg concentration on 8 day application to 21 days old tobacco seedlings. Their results indicated 13% 26%, 12% and 13% reduction in the shoot length respectively. Hosokawa et al (1984) have reported the inhibitory effect of tridemorph, triparanol and triarimol, on steroid metabolism. The results of their studies indicated the inhibition of sterol metabolism at various steps by these compounds in different systems such as carrot, tobacco and soybean cell cultures and they have correlated this inhibition as the reason for the growth inhibition of plants. Bach (1985) has used a group of enzyme inhibitors of sterol biosynthesis such as A 25822 B, U 18666 A, MER 29. iminosqualene, clotrimazole, miconazole, triarimol, naftifine and deoxycholate and came to the conclusion that all these compounds inhibit sterol biosynthesis at some steps and the alteration or reduction in the sterol leading to the growth inhibition of radish seedlings. In his studies using radish seedlings he observed that by 10th day A 25822 B (5 ppm), iminosquelene (5 ppm), Triarimol (50 ppm) U 18666 A (40 ppm), clotrimazole (20 ppm) naftifine (100 ppm) triparanol (10 ppm) miconazole (10 ppm)

deoxycholate (200 ppm) inhibits the growth of seedlings to about 45%, 50%, 25%, 15%, 25%, 35%, 20%,60% and 30% of the control respectively. Grossmann et al, (1984) have brought forth evidence for the absolute necessity of sterols for the cell division and growth of cell cultures of maize and soybean. They studied the effect of added sterols in recovering the inhibition of cell division caused by tetcyclasis which acts by way of modifying the cyclopentano phenanthrene ring system of sterols. Fletcher et al (1986) studied the effect of different triazol derivatives which affect sterol biosynthesis. Their studies with different compounds on 28 day application to Kentucky blue grass showed different degrees of inhibition of growth. They showed that 50 mg/l concentrations of tridemephon, triademinol, S-3308, S-3307 and palcobutrazol can reduce the stem length by 75%, 62%, 67%, 19% and 40% of the control by 28th day of application and they have correlated this inhibition of growth to the reduction of sterol content. Burden et al (1987) studied the effect of 9 different sterol biosynthetic inhibitors on barley seedlings and showed that application of 20 mg/l conc of 1-RS, 2-SR triademinol, 1-RS, 2-RS triademinol, 1-RS 3-SR Palcobutrazol, 2-RS, 3-RS palcobutrazol, nuarimol, triapenthenol,tridemorph, fenpropimorph and prochloraz can reduce the growth to 48%, 65%, 29%, 30%, 32%, 27%, 57%, 50%

& 80% of the control respectively as a result of reduction in the sterol content. Haughan et al, (1987) studied the growth inhibition of celery cell cultures by 50 μM of palcobutrazole and showed that the dry wt. of the cell cultures was reduced to 18 of the control by 21st day of incubation.

There are not many reports on the growth and sterol biosynthesis inhibition caused by mevinolin. Bach & Lichtenthaler (1983) studied the inhibition of elongation growth in radish and inhibition in sterol biosynthesis. They observed an uninhibited growth of roots to a length of 20 mm which was not blocked even by 10 um of mevinolin. In the present investigation pronounced reduction in the root growth was observed only at concentrations of mevinolin above 50 um. Thus Solanum seems to be more resistant to mevinolin compared to radish. This resistance may be due to the fact that Solanum plants have quite an active isoprenoid synthetic activity than cruciferous members which are known to have active glucosinolate metabolism derived from amino acids. The inhibition of elongation growth by mevinolin was more pronounced in dark conditions. This observation is similar to the observation of Bach & Lichtenthaler (1983) as they showed that the growth inhibition of the seedlings was recoverable by the addition of MVA.

The inhibition of accumulation of sterols and steroidal alkaloids by mevinolin observed in our experiments is in agreement with the observation of Bach & Lichtenthaler (1983, 1987). Experiments with radish seedlings have shown more than 50% inhibition in sterol synthesis by 5 μM mevinolin. But in our experiments mevinolin at conc. above 50 μm could bring about such a drastic effect on sterol biosynthesis. In cell cultures system of S. xanthocarpum mevinolin at 25 μM concentration induced more than 50% reduction in the sterol content and growth. Döll et al., (1984) have also reported a similar reduction in the sterol biosynthesis and growth when worked with cell cultures of Silybum marianum.

From the foregoing it is clear that inhibition of growth of various tissue systems of S. xanthocarpum by mevinolin may be attributed to the inhibition of synthesis of isoprenoid compounds such as natural cytokinins, gibberellins and sterols. However, the hormone synthesis is restricted to plastids and plastid membranes are comparatively very less permeable to mevinolin. Moreover, hormones are active at very low concentrations and the blockage of this syntehsis requires very high conc. of mevinolin. Therefore, the observed inhibition of growth by low concentration of mevinolin can be attributed to the reduction in the sterol synthesis.

The first committed precursor of isoprenoid pathway, mevalonate when added at concentrations upto 4 mM in the case of cultured leaf discs (Fig.12) and upto 4 mM in the case of cell cultures (Fig. 13) was able to reverse the mevinolin induced inhibition to near normalcy. Bach & Lichtenthaler (1983) have demonstrated the recovery from the 0.25 μM mevinolin induced inhibition of root growth by 2 mM of MVA alone or in combination with 30 μM GA_3.

The exogenous squalene at 1 μM could bring about 23% recovery of the mevinolin induced growth inhibition of cell cultures (Fig.15). Maximum reversal of inhibition was achieved when squalene was added upto 1.5 μM in the case of cultured leaf discs. Exogenous squalene at a concentration 1 μM in the case of 25 μM mevinolin treated cell cultures and 1.5 μM in the case of 10 μM mevinolin treated cultured 10 mm leaf discs of S. xanthocarpum showed maximum recovery from the mevinolin induced inhibition of growth and sterol biosynthesis (Fig14 & 15). Ceccarelli & Lozenzi (1984) have shown that 0.1 to 0.5 mM of farnesol when supplied alone or in combination with 0.2 mM of MVA recover from the 5×10^{-5} M mevinolin induced inhibition of growth of callus cultures of Helianthus tuberosus. Hashizume et al (1983) and Ceccarelli & Lorenzi (1984) used a similar inhibitor (compactin). Hashizume et al's studies showed that the

inhibition of tobacco callus growth caused by mevinolin cannot be reversed by exogenous acetate. The studies on sunflower callus cultures by Ceccarelli & Lorenzi showed that farnesol at 0.1 to 0.5 mM can cause reversal of the compaction induced growth inhibition. Subramani (1991) has also showed the recovery of sterol biosynthesis to a greater extent by the application of squalene when sterol synthesis of Solanum cell culture was inhibited by SKF 525-A. This inhibitor blocks the cyclization step in the formation of perhydrocyclopentano phenanthrene ring system in sterol synthesis.

Large quantum of data are available elsewhere on the efficiency of exogenous sterols in recovering the growth inhibition caused by sterol biosynthesis inhibitors. (Paltauf et al 1982; Berg et al, 1983; Grossmann et al, 1985; Kawasaki et al, 1985; Nitsche et al 1985; Haughan et al, 1987; Tatan et al, 1988; Burden et al, 1989). In our experiments however only cholesterol was used to reverse the inhibition. Grossmann et al, (1985) have shown that cholesterol is the most effective sterol in recovering the sterol biosynthetic inhibition caused by tetcyclasis,. Kawasaki et al (1985) have shown that ergosterol to be more effective compared to cholesterol to restore the growth in the mutant yeast GL-7 which is unable to carry out normal

sterol metabolism. Burden et al (1989) have cited many instances of recovery of growth by added cholesterol when sterol synthesis was blocked by various inhibitors. Absolute structural specificity of sterols in such transformation have also demonstrated in Saccharomyces by Pinto and Nes (1985). This absolute sterol specificity and its requirement is because of the role played by sterols as the functional and architectural component of membrane systems of the cells as proposed by Nes and Mc Keen (1977) and Nes and Heftman (1984).

Similarly the mevinolin treated seedlings of Solanum xanthocarpum exhibited severe inhibition of growth and sterol metabolism. As shown in the case of animal systems (Sabine, 1983) and in plant systems (Bach and Lichtenthaler,1983) the gateway enzyme HMGR is highly inhibited by mevinolin. As an after effect of inhibition of HMGR there will be a considerable reduction in the flux of mevalonate in to higher order intermediates of sterol pathway. Reduction of the in vivo synthesis of gibberellin, sterols and other physiologically important isoprenoid compounds can be the reason for the observed inhibition of the root growth. Sterols are found to be essential for normal cell division (Grossmann et al, 1985) and membrane functioning (Grunwald, 1978). As suggested by Bach and

Lichtenthaler (1983), a possibility that the decrease in the endogenous cytokinin may be the reason for the reduction in hypocotyl length. They have pointed out the development of sun type chloroplasts as in the case of cytokinin treated plants upon treatment with mevinolin.

Characterization of the selected cell lines of *Solanum xanthocarpum* resistant to mevinolin.

Mevinolin resistant cell lines of *S. xanthocarpum* were isolated using the cell plating technique described by Bergman (1960). Cell lines which were resistant to LD 50 concentration was selected as it is the desirable inhibitory concentration to obtain variant cell lines of use. This is because of the fact that higher concentrations of the inhibitor can kill even the desirable variant population, and lower concentrations may not be effective in screening off the physiological mutants. Moreover, at this concentration biochemical changes responsible for the principal mode of action would be clearly expressed (Burden *et al*, 1989).

Before these selected colonies were subjected to characterization they were grown in the selective medium for at least 10 to 12 subcultures. This treatment in turn helped in eliminating the physiological mutants to a great extent

and to stabilize the mutations (Berlin and Sesse. 1985). In order to characterise the variant cell lines, different criteria were used such as (i) their ability to grow on selective medium, (ii) sterol and steroidal alkaloid contents in the selected line, (iii) study of the rate of incorporation of labelled acetate into the cell lines in the presence and absence of mevinolin and (iv) comparative analysis of HMGR activity in the resistant and unselected/ control lines.

Mevinolin resistant and wild type cell lines of S.. xanthocarpum were grown in batch cultures under selective and non selective conditions. Under non selective conditions there was no difference in the growth of these two cell lines.

Under selective conditions the mevinolin resistant cell line showed same growth as that observed under non stressed condition. Uninhibited growth in the presence of selected cell line is shown to be a criterion for resistance by many workers. Hibberol et al, (1980) showed that the lysine threonine feed back resistant cell lines of maize can grow on 2.5 mM conc. of lysine and threonine. Shimamoto and Nelson (1981) showed the growth of aminopterin resistant cell lines of maize to grow on 25 µM/ml conc. of aminopterin. Redway & Vasil (1990) selected S-(2-aminoethyl)

L-cysteine tolerant cell lines of Pennisetum and showed that this cell line can grow on 0.5 mM AEC as the resistance characteristic. Subramani (1991) obtained resistant cell lines of S. xanthocarpum and S. dulcamara resistant to the drug SKF-A-525 at conc. of 0.08 mg/ml and 0.06 mg/ml. He used the leaf disc assay as well as growth of the cell line under selection pressure as the criteria for resistance.

Though there are many reports on selection of cell lines resistant to antibiotics, (Maliga et al., 1977 ; Sceplo and Maliga, 1982), amino acid resistance (Hibbereol & Helson, 1981; Redway & Vasil, 1990) the stability of the resistance is not proved by the analysis of the plantlets obtained. Flick, (1983) has pointed out certain criteria like 1) low frequency of occurrence 2) stable when grown in the absence of the stress 3) stability through regeneration and 4) heritability of mutation for a resistance to be stable.

Therefore, in my investigation the characterization of the resistant cell line was checked using a set of criteria like comparison of the growth rate of selected and wild type cell line in selective and nonselective medium, comparison of rate of synthesis of sterols by the wild type cell line with that of resistant cell line, comparison of the activity of the enzyme in question (HMGR) in the selected and

nonselected cell lines. Alferman et al (1975) have used the over production of anthocyanin to be a criterion for the variance. Widholm, (1977) has also used the over production of aminoacids as the criterion for resistance. Similarly Redway & Vasil (1990) used the over production of lysin as the criterion for resistance.

Treatment of the cell culture with inhibitors of the metabolic pathway has shown to increase the enzyme activity in question when the inhibitor is blocking a specific enzymatic step (Lea and Norria, 1976; Berlin, 1982; Chin et al., 1982). However, there are no reports on the constitutive induction of such enzyme systems of sterol biosynthetic pathway from the plant system. Chein et al., (1982) have shown 500 fold induction of HMGCOA reductase enzyme when the CHO cells were grown under the selection pressure 40 μM compactin which is an inhibitor of HMGR. In the present investigation the increase in the sterols and steroidal alkaloid contents, and the higher rate of incorporation of the ^{14}C acetate into steroids in the selected resistant cell line over control is a clear indication of stability of resistance to mevinolin.

Though the observed overproduction of steroids was stable even up to 12 subculture cycles, the genetic analysis

of the regenerants is required to unequivocally prove the genetic basis of this observed variation. As only when these regenerants reproduce sexually and transmit this character, it can be definitely stated to be a genetic mutation (Carlson, 1975).

In an attempt to determine the reason for the higher rate of incorporation of the ^{14}C acetate in to the sterol fractions and thus to account for the increased accumulation of the steroids in the selected cell lines of Solanum, relative activities of HMGR, the gate way enzyme of the mevalonate pathway was studied. The results indicated a 2.5 fold increase in the activity of the enzyme. However, dependence of enzyme on NADPH or DTT was not studied as the question before the investigation was the difference in the activity of wild type and variant cell lines.

Most of the regulational as well as physiological aspects of HMGR enzyme come from studies on animal systems and not much is done to the understanding of plant HMGR. This painful situation resulted because of the relatively low specific activity of this enzyme in plants (Gray, 1987).

Some of the aspects of plant HMGR is studied in the recent years Brooker and Russel,1975a, and b, 1979; Grumbach

and Bach, 1979; Bach et al, 1980; 1986; Bach and Lichtenthaler, 1984; Camara et al, 1983; Arebalo and Mitchell, 1984; Maurey et al, 1986; Oba et al, 1986; Wititsuannakul 1986; Skrukrud et al, 1987; Vogeli and Chappel, 1988; Chappel et al, 1989).

Studies on this enzyme has revealed the enzyme regulates the latex production in Hevea brasiliensis (Wititsuwannakul,1986). Bach et al (1986) have suggested a possibility of the regulation of sterol biosynthesis in plants by controlling the mevalonate flux by HMGR

Optimum pH for plant HMGR is reported variously differing from system to systems. From the experiments with radish seedling enzymes (Bach and Lichtenthaler, (1984); Bach et al, 1986) the pH optimum was found to be 7.5. Sipat (1982) has reported pH optima 6.1 to 6.9 in Hevea latex enzyme. Brooker and Russel (1975) found that HMGR activity was optimal at pH 6.9 when 50,000 g membrane fractions of pea seedling was used as the enzyme source. Suzuki et al reported a pH optimum of 7.3-7.5 for 105,000 membrane fraction enzyme for sweet potato roots. Reddy and Ramdas (1986) have shown maximal activity of cytosolic HMGR of guayule is at pH 7.00 while plastidic HMGR has a pH optimum of 7.5. Optimum pH 7 is reported for tobacco which belongs to

the same family solanaceae (Vogele and Chappel, 1988; Chappel et al, 1989). From the results of these earlier works it is clear that pH requirement of HMGR varies from plant to plant.

The activity of HMGR in plants is shown to vary from plant to plant as is evident from the reported Km values for RS.HMG CoA. 16,000 g membrane fractions of radish seedlings showed a Km of 5.5×10^{-6} M for HMGCoA (Bach & Lichtenthaler, 1984) Hevea latex enzyme showed km of 5.6×10^{-5} M (Sipat, 1982 a) sweet potato roots enzyme showed km of 1.3×10^{-6} M(Suzuki et al, 1982) tobacco cell cultures showed 1.8×10^{-5}M. (Chappel et al, 1989). However, the inhibition of this enzyme by 10 nmol mevinolin is in accordance with the previous reports (Bach and Lichtenthaler, 1982; Bach et al, 1984; Murey et al, 1986). The observed 2.5 five fold increase in the activity of the enzyme in the selected cell lines may be due to the adapted modulation of the enzyme system or due to the production of more enzyme molecules as a result of mutation. Similar increased (500 fold) activity of the UT1, cell line resistant to 40 μM compactin treated Chinese hamster ovary cells was observed by Chin et al (1982).Mutational nature of UT1 cells was determined by Chin et al (1982) from the analysis of enzyme concentration in the selected cell line

(UTI) and changes in the internal structure of the UTI cells and DNA analysis. In the present investigation only continued activity of the cell line was checked to determine the mutational nature. Unlike the observation of Maurey et al (1986) that 10 μM mevinolin treatment of Ochromonas cells showing a 10-15 fold increase in the HMGR activity and a decline to normal levels of activity in two hours of removal of mevinolin stress, the activity of HMGR was 2.5 fold more than the control cell culture even when the selected cell line was grown on mevinolin minus medium. This higher activity of HMGR is suggestive of amplification of the enzyme.

Incorporation of ^{14}C acetate in to sterols:

In order to see whether the increased contents of sterols in the selected line is due to the increased rate of synthesis of sterols or reduced rate of degradation, rate of incorporation of 2-^{14}C acetate into sterols in the selected and wild type cell lines were compared. As shown in the table (16) the incorporation of acetate in to the selected cell line was 1.32 to 1.76 fold greater in to the sterols. When the unselected cell line was incubated in presence of 10 μM mevinolin the incorporation of ^{14}C into the wild type cell line fell down to 60 to 70%, of the control values. In the case of selected cell line the

incorporation was more than that of the control when incubated in the absence of mevinolin. Comparatively the incorporation in the selected cell line was 1.29, 1.51, and 2.02 fold more than that of the wild type cell line. Similar increased incorporation of 2-^{14}C acetate in to the variant cell line of Solanum selected against SKF 525 A is on record (Subramani, 1991).

Characterization of the Regenerated variant plants

Characterization of the regenerated variant plant was carried out using leafdisc assay as well as by the determination of steroidal levels in the young leaves. As shown in the (Table 17) there was no apparent loss of weight like the control leaf discs in the case of plant No. I and II. Moreover these plants showed significantly high amount of steroids in the plant parts.

Best system for variant selection is plant protoplasts but use of cell culture is commonly used as it is easy to handle in practice. About 188 variant cell lines have been isolated and characterized in the case of maize (Flick, 1983). This resistance selection is carried out at sublethal dose of inhibitor or analogue (Hartman et al,1984 b). Though variant cell lines and in some cases variant plants are obtained few of them are characterized biochemically and

only very few of them are genetically characterized (Berlin & Sasse, 1985;Conner & Meridith,1989).

Though many variants which are resistant to amino acid analogues (Widholm, 1977, a; Redway & Vasil, 1990) herbicide resistance, drug resistance etc. (Berlin & Sasse 1985; Conner & Meridith 1989), there are no reports on selection of variant plants which differ in steroid composition other than that of Subramani (1991). Though Chiu et al (1980) tried selection for variance with respect to sterol composition in Nicotiana tabacum var. wisconsin no conclusive data on the type of variance observed is available.

In the present investigation, the regenerant variants are characterized with leaf disc assay as well as steroidal content determination. As shown by Subramani (1991) the variants showed a mosaic of true and false variants i.e. some of the variant plants derived from the selected cell lines of Solanum showed resistance to LD 50 concentration of the inhibitor (Mevinolin) while one did not. The steroidal analysis showed a close correlation to the resistance i.e. higher content was associated with stability of resistance trait.

Subramani (1991) selected the cell lines of S. xanthocarpum & S. dulcamara resistant to the drug SKF 525-A which blocks the isoprenoid pathways and analysed the regenerants. Out of a dozen of these plants analysed only 4 of them were true resistants while others were escapes of the selection or physiological variants so that they failed to express this trait in the regenerated plants.

Present observation shows that the high amount of steroids present in the variant callus culture is not expressed in the variant regenerants. Similar observation is on record when selected for SKF 525-A (Subramanii 1991). This difference may be due to the development related regulation of the steroid pathway.