PART B
INTRODUCTION

It is fundamental that for sustained growth in culture, as in the whole plant, continued cell division is required. These cells, when arrested in $G_1$ or $G_2$ phases of the cell cycle, start differentiating (Van't Hof, 1985). Thus, proliferation and differentiation states can be mutually exclusive in plants (Martinez et al. 1992). Moreover, cell division in plants is localized to the meristems and reflects a high degree of flexibility in its intrinsic controls during the course of its iterative development.

Recent advances in the study of control of cell division have begun to yield an insight into the mechanism of processes that regulate morphogenesis and differentiation in plants (Hirt et al. 1992; Hemerly et al. 1993, 1995). Isolation and characterisation of many regulatory genes intimately involved in cell division cycle in plants have been reported in the past couple of years (Jacobs, 1995). Characterization of these cell cycle related genes can provide the genetic basis of how spatial and temporal controls may be linked (Hemerly et al. 1995).

The molecular and biochemical mechanisms of cell cycle control in plants appears to be largely similar to other eukaryotes (Ferreira et al. 1994; Staiger and Doonan, 1993; Francis and Hallford, 1995). But the constraint imposed by rigid cell wall and the comparative lack of mobility in plants, establish a unique developmental context in which cell division must be controlled. The spatial and temporal control of cell division is believed to be directly responsible for generating new structures and morphogenesis throughout development (Hemerly et al. 1995). Although the overall growth of plants has an autonomous control, signals are involved in coordinating growth throughout the development of plant and to shepherd cells from one cell cycle phase to the next.

It is known that hormones play a vital role in promoting and inhibiting plant growth.
Hormonal activation of transcription of some cell cycle control genes would provide tools for examining the influence of growth hormones in mitotic activity. Isolated protoplasts can be stimulated to divide if provided with auxins and cytokinins. Under these circumstances burst of cdc2 expression follows the initiation of cell division (Martinez et al. 1992). Transcription of the cdc2 in carrot has been correlated with the proliferative capacity of auxin induced de-differentiated cells (Gorst et al. 1991). But still very little is known about the mechanism of action of plant specific mitogens.

Glyoxalase I is an enzyme which is not directly involved in the metabolism of the cell, but is found to be associated with proliferation in cultures of pea, Datura, Amaranthus, soybean and Brassica (Ramaswamy et al. 1983; Das et al. 1987; Sethi et al. 1988). It catalyses the conversion of methylglyoxal, a keto-aldehyde, to its respective thioester S-lactoylglutathione (Thornalley, 1990). Due to its close correlation with cell division, glyoxalase I is employed as a marker enzyme to determine the extent of proliferation. The exact mode of association of glyoxalase I with cell division is not yet known. However, it is postulated that its involvement with cell division is either by removing methylglyoxal, a cell division inhibitor from the system or by producing S-lactoyl-glutathione, which plays an important role in microtubule assembly (Gillespie, 1975).

In the previous section, we have seen the mechanism of development at the tissue level. But to understand the cellular mechanism of differentiation, single cell cultures or protoplast cultures are most suitable. Tobacco protoplasts have been well standardized for cell cycle studies (Galbraith et al. 1983). Thus, from the growth controls we move on to the cell cycle controls in plant systems.

Present investigations were attempted with the following objectives:

a. To understand the cell cycle status of mesophyll protoplasts during early period of regeneration.

b. To understand the interaction of phytohormones with division of protoplasts and glyoxalase I activity.
Knowledge of control of cell division in eukaryotes has increased considerably in the recent years. The first appreciation of the cell cycle as consisting of four distinct phases was made in root tips of *Vicia faba* (Howard & Pelc, 1953). Despite this initial lead from the plant system, the lack of powerful genetic models such as yeast or of a well characterized biochemical system such as the *Xenopus* oocytes, has hindered progress of the understanding of cell division. Nevertheless, the mechanism of operation of plant cell cycle is very similar to that of other eukaryotes (Ferriera *et al.* 1994). This observation leads to almost instinctive prediction that many of the molecules involved in controlling cell division described in other systems should, first, be present in plants, and second, have similar function as their yeast and animals counterparts.

In biochemical terms, the cell cycle is a phosphorylation cascade which enables cells to enter mitosis. In structural terms, the cell cycle represents a series of check points which ensures that cell size and environmental conditions are optimal for cell division (Lyndon, 1990).

### 2.1 Control of cell proliferation during plant development

Cell division in plants serves to subdivide space in the expanding organism rather than to supply cellular building blocks, as it does in animals. Despite these contrasting developmental contexts, transition through and between stages of the mitotic cell cycle are controlled in both kingdoms by related families of cyclin-dependent kinases (CDKs) and their regulatory subunits (cyclins) (Francis and Halford, 1995). Passage of animal cells through G₁, S and G₂ phases of the cycle is controlled by complexes of CDKs, cyclins, transcription factors, tumour suppressor proteins and inhibitor proteins. As the network of interacting genes and proteins regulating the yeast cell cycle starts becoming
clear, and homologous of some of the genes identified; plant molecular and cell biologist began to ask whether a similar system exists in plants.

However, if comparisons are restricted to the control of proliferation, a few dissimilarities can be listed.
1. Plant cells can quit cell cycle and differentiate either in $G_1$ or in $G_2$.
2. Upon stimulation, most mature plant cells can dedifferentiate and re-enter the cell cycle, regardless of being in $G_1$ or $G_2$.
3. Correlation of $p^\text{cdc34}$ mRNA levels with proliferative state is not so conclusive in plants as in animals.
4. Animal mitotic cyclins are subdivided as A and B types cyclins according to their sequence differences and expression patterns but no such distinction exists in case of plants (Savoure et al. 1995).
5. A clear state of quiescence (comparable to $G_0$ in animals and quiescence in yeast) has yet to be demonstrated (Ferriera et al. 1994)

A close examination of processes in animal systems where cell cycle progression is triggered by the activation of a limiting factor yields three immediate candidates: cyclins, cdc25 and MAP kinase. A detailed examination of spatial and temporal patterns of expression of Arabidopsis cyclins showed -
1. Expression restricted to zones of mitotic activity and is not induced or present in situations that reflect a competence for cell division such as wounding or apical meristem of dark grown seedling (Hemerly et al. 1993).
2. In root apical meristem, where rate of cell proliferation is not high - cdc2 expression is cell autonomous reflecting the distribution of cells in $G_2$ (Ferriera et al. 1994).
3. Cyclin expression is an early event during de novo induction of cell cycle, e.g. lateral roots formation from pericycle.
Therefore pattern of expression of cyclin genes is consistent with the hypothesis that expression of cyclin is responsible for activation of cdc2 kinase and consequently induction of cell division.
2.2 Flowcytometric characterization of plant protoplasts

Flowcytometry (FCM) offers a convenient and accurate tool to study cell cycle in single cell suspension cultures. But due to the three dimensional structure of plants, owing to the rigid cell wall, this technique could be limited to protoplasts only. The instrument needs to be improvised still for plant tissues - (i) larger size (100 μ) aperture and (ii) choice of fluorochrome which does not overlap with the inherent fluorochromes of plant cells (Galbraith et al. 1983; Bergouniox et al. 1988). FCM has been useful in obtaining information on -

(1) characterization of differentiated tissues according to relative frequency of nuclei having \( G_0/G_1, G_2 \) and \( S \) levels of DNA.

(2) Quantification of DNA levels in \( G_0/G_1 \) to establish ploidy of DNA variability in different tissues.

(3) Studying nucleic acid synthesis during regeneration of protoplast to whole cells and their subsequent division under hormonal control.

2.3 Plant growth regulators

Hormones have a special place in the subject of plant development. Much of the work on plant development by physiologist has concerned the nature, localization and effects of these growth regulators. There are major questions about the role of hormones that remain unanswered.

Auxin, the first plant hormone to be discovered is generally identified as the growth stimulating substance. Auxins have been a key factor in many classical studies of plant development. Many processes depend on its presence: cell elongation, phototropism and gravitropism, xylem differentiation, apical dominance. Biochemical analysis has revealed several auxin binding proteins (Palme et al. 1992) of which the best characterized can apparently act as a receptor and initiate signal transduction (Goldsmith, 1993). Auxin activity, mediated by as yet uncharacterized pathways, results in rapid changes in gene expression (Takahashi, 1995). However, it remains to be seen why auxin has different
The Glyoxalase System
effects on different cells.

Although cytokinins were discovered by their ability to induce cell division, they are now known to act in combination with other phytohormones to regulate diverse responses in plants, including seed germination, *de novo* bud formation, release of buds from apical dominance, leaf expansion, reproductive development and senescence (Brzobohaty *et al.* 1994).

2.4 The glyoxalase system

One enzyme that has been shown to be induced by auxin and to correlate with cell division is glyoxalase I. Glyoxalase system catalyses the conversion of methylglyoxal to D-lactate via the intermediate S-D-lactoylglutathione. It comprises of two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione (Racker, 1951; Mannervik, 1980; Murata *et al.* 1989; Thornalley, 1990).

Glyoxalase I (EC 4.4.1.5), catalyses the formation of S-lactoyl glutathione from the hemithioacetal formed nonenzymatically from methylglyoxal and reduced glutathione (Mannervik, 1980). The glyoxalase system is present in the cytosol of prokaryotic as well as eukaryotic cells. The wide spread distribution and presence of glyoxalase system in living organism suggest that it fulfils the function of fundamental importance to biological life. Throughout eighty years of study biological function of glyoxalase has remained unclear.

In 1951, Racker established for the first time that the glyoxalase pathway involved two discrete and consecutive reaction steps. Thereafter, studies on the effect of methylglyoxal on the biological system found associated antiviral activity (Underwood and Weed, 1952) and antitumour activity (Szent-Gyorgi, 1965). Gillespie (1975) suggested that the glyoxalase system may regulate the assembly of microtubular cytoskeleton, mediated by S-D-lactoylglutathione.
Historically, experimental evidence has emerged for an important function of the glyoxalase system in: (i) the detoxification of methylglyoxal (Egyud and Szent-Gyorgi, 1968), (ii) a glycolytic bypass for the glyceraldehyde-3-phosphate to the pyruvate section of the Embden-Meyerhof pathway and (iii) the control of cell proliferation (Gillespie, 1975).

Glyoxalase I was demonstrated in higher plants e.g. pea in which the enzyme activity increased with the age of the seedlings and was stimulated by exogenous application of IAA (Paulus et al. 1993). The glyoxalase I activity was higher in meristematic region as compared to the elongation zone of the roots (Ramaswamy et al. 1983). In callus cultures of Datura, glyoxalase I activity increased with the age of the culture, accompanied by increase in DNA and protein synthesis and decreased with the addition of mitotic inhibitors, vinblastine and methylglyoxal and specific glyoxalase I inhibitors, isoascorbate and squaric acid (Ramaswamy et al., 1984). Studies in Amaranthus have implied the role of glyoxalase I in cell proliferation (Das et al. 1987). Glyoxalase has been implicated with cell proliferation in response to pH changes, polyamines and red light, whereas glyoxalase levels decline in differentiating cells induced by inhibitors of polyamine and ethylene biosynthesis, cell cycle blockers and far red light (Basu et al. 1988; Sethi et al. 1988; Bagga et al. 1987).

It is clear that the cell cycle activity is intimately connected with differentiation and that an understanding of plant cell cycle control is central to the establishment of different patterns of plant development.
MATERIAL AND METHODS

3.1 Plant material

Shoot tip culture of *Nicotiana tabacum* var SR1 maintained in our laboratory on MS basal salts (Murashige and Skoog, 1962) with B5 vitamins (Gamborg *et al.* 1968) were employed as experimental material.

3.2 Isolation of protoplasts

Fully expanded leaves of six week old sterile shoot cultures of *Nicotiana* were used. Protoplasts were isolated according to the procedure of Potrykus and Shillito (1986). Leaves were teased in solution of CPW salts (*Table 1*) containing 13% mannitol, 1.5% cellulase and 0.05% macerozyme (pH 5.8) and incubated in dark for 16-18 h at 25°C without shaking. Protoplasts were squeezed out in CPW containing 21% sucrose and purified by passing through steel mesh followed by centrifugation for 5 min at 80Xg (Jouan C500 bench centrifuge, swing out rotor). The protoplast count was taken on a haemocytometer and viability of protoplasts was checked by fluorescein diacetate (FDA) staining (*Fig. 1*).

FDA (5mg/ml acetone) was added to CPW13M medium with dilution of X1000. Equal volume of FDA solution was mixed with the dense protoplast suspension in culture medium and examined under fluorescence microscope (Nikon, Japan) after 5 min.

% Protoplast viability = \[ \frac{\text{No. of fluorescing protoplast}}{\text{Total no. of protoplast}} \times 100 \]
Fig. 1 Protoplasts isolated from *N. tabacum*.

A. Protoplasts without staining.
B. FDA stained protoplasts.
(Magnification X100)
Table 1

Composition of CPW salts (mg/L final concentration)

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>101</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1480</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>246</td>
</tr>
<tr>
<td>KI</td>
<td>0.16</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.025</td>
</tr>
</tbody>
</table>

3.3 Short term treatments

After protoplast isolation, hormones and inhibitors were added to the culture medium for 5 min. Protoplast were washed twice with fresh culture medium and incubated for 20 h. Cell number and glyoxalase I activity were measured from these samples.

3.4 Glyoxalase I activity assay

The procedure has been described in Material and Methods, Part 1 (3.5.1)

3.5 Cell cycle analysis by flow cytometry

Sample preparation: Protoplasts (1X10⁶ cells per ml) were fixed in 70% ethanol (final vol.) at an interval of 6 h for a period of 30 h. Cells were pelleted down by centrifugation at 1,000 rpm for 5 min at 4°C. The pellet thus obtained was rinsed with Triton X-100 (final concentration 1%). After RNase treatment, cell wall material was removed with a 10μm mesh nylon filter and further organellar debris eliminated by pelleting the nuclei with a 110Xg centrifugation for 5 min and repeated twice. Immediately the pellet is resuspended in P medium (Table 2) (Bergounioux and Brown, 1990). Nuclei were stained by propidium iodide (50 μg/ml final concentration) and incubated for 20 min before analysing on the flow cytometer.
Table 2

<table>
<thead>
<tr>
<th>P medium (pH 6.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Macro elements (g/L)</td>
</tr>
<tr>
<td>NH₄NO₃</td>
</tr>
<tr>
<td>KNO₃</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b. Micro elements (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO₄</td>
</tr>
<tr>
<td>H₃BO₃</td>
</tr>
<tr>
<td>MnSO₄.5H₂O</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
</tr>
<tr>
<td>AlCl₃.6H₂O</td>
</tr>
<tr>
<td>KI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>c. Sorbitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>126 g/L</td>
</tr>
</tbody>
</table>

Flow cytometry: Propidium iodide stained nuclei were analysed on EPICS V cytofluorometer (Coulter, FL, USA) fitted with an argon laser (Spectra-Physics 2025-25) and 250 μm flow tip. The total number of events analysed for a set was 10,000. An event is described as a particle passing through the flow cell and intercepting the laser. Each experiment was repeated at least thrice and two replicates for each sample were used. The outputs were gated manually to accommodate uniformity in analysis of different type of events. The resulting histograms were analysed by 'multicycle' (Phoenix flow system, USA) univariate deconvolution program. Protoplasts treated with colchicine for 24 h were used as standard for calculating the diploid DNA content.

3.6. Statistical analysis

Data presented are the average of three or more experimental values. The significance of difference between the data pairs was evaluated by analysis of variance (ANOVA).
RESULTS

4.1 Cell cycle analysis of mesophyll protoplasts of tobacco

Propidium iodide stained nuclei were analysed on EPICS V cyto-fluorometer using 100 mWatt argon laser. The output profiles were manually gated to select the best population of cells with respect to their size, granularity and stain adsorbance. The resulting histograms were analysed by using the ‘Multicycle’ software program. The various sub-populations of nuclei were identified as G₁, S and G₂/M stages on the basis of their differential DNA content (Table 1). From these studies on the mixed population of protoplasts, it was found that at T₀, 51.6% were in the G₁ phase and there was 30% synchrony in the protoplasts undergoing division. Protoplasts retained their capacity to divide till 30 h of culture without supplementing isolation medium with any growth regulator. Most of the cells pass through G₂/M phase after 24 h of culture. After 30 h, cells were arrested in G₁ phase.

Table 1: Distribution of protoplasts in different phases of cell cycle at different times of culture.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Time (h)</th>
<th>G₁</th>
<th>S</th>
<th>G₂/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>51.6±0.55</td>
<td>41.0±0.81</td>
<td>6.6±0.21</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>55.9±1.25</td>
<td>44.0±0.61</td>
<td>0.1±0.08</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>40.5±0.69</td>
<td>59.0±0.95</td>
<td>0.4±0.08</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>36.9±0.42</td>
<td>62.1±0.93</td>
<td>0.9±0.06</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>29.7±0.73</td>
<td>41.0±0.76</td>
<td>29.4±0.35</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>99.7±0.46</td>
<td>0.1±0.08</td>
<td>0.2±0.06</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E of at least three experiments. Values are given in percentage.
Fig 2  Schematic representation of operations on Flow cytometer to optimize and characterize the events for analysis of cell cycle.

A. Forward scatter v/s side scatter plot for the total number of events without gating.
B. Propidium iodide fluorescence (i.e. DNA content) v/s cell count.
C. Same as B but after gating (A).
D. Scatter plot of DNA content v/s fluorescence peaks (depends on granularity of the nucleus).
E. Histogram of plot D without gating.
F. Histogram of plot D with gating.
G. Scatter plot refined further by another gating.
H. Final output histogram for ‘Multicycle’ software analysis.
Fig 3  An output from 'Multicycle' deconvolution program depicting distribution of colchicine treated protoplasts in different phases of cell cycle.
4.2 Cell division and glyoxalase I activity

After protoplast isolation, cell count and glyoxalase I activity were monitored till 30 h at an interval of 4 h. Approx. 6X10^6 cells were used for measuring enzyme activity as described by Ramaswamy et al (1983). The protoplasts stabilise for some time and show a lag phase till 12 h (Fig. 4). The logarithmic cell division occurs between 12-24 h. Glyoxalase I levels are also low in the initial period and a rise in activity is observed between 12-18 h, after which the activity falls. Thus an increase in cell number is preceded by an increase in activity of glyoxalase I.

Isoascorbate, an inhibitor of glyoxalase I, was able to lower the rate of cell division by 28% (Table 4). Colchicine- the cell cycle blocker, also decreased glyoxalase I activity by 45% even during the phase of rapid cell division.

4.3 Effect of hormones on protoplast division and glyoxalase I activity

Effect of auxin and cytokinins were studied separately as well as collectively on the cell number and enzyme activity. Protoplasts after isolation were cultured on media: (i) containing no hormones (Control), (ii) supplemented with NAA (1 ppm), (iii) supplemented with BA (1 ppm) and (iv) supplemented with NAA (1 ppm) and BA (1 ppm). The enzyme activity and cell number were monitored for the first cell cycle (approx. 28 h) at an interval of 4 h.

It was found that in protoplasts cultured on NAA alone, cell division was approx 18% more than those cultured on NAA + BA, during the log phase of cell division (Table 2). BA too increased the cell count (13% more) over the control but was significantly less than those cultured on NAA.

Similarly, NAA induced highest glyoxalase I activity (20% more over the control) after 20 h of culture (Table 3). Whereas, BA treated samples had low level of glyoxalase
Fig 4 Growth kinetics and enzyme activity of glyoxalase I in Nicotiana tabacum protoplast. Data represent the mean value of three different experiments. SD < 1%.
Table 2  Effect of hormones on growth kinetics of *Nicotiana* protoplasts.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cell Number (1 X 10^6 cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in parenthesis indicate % difference, taking the control value as 100.

* * indicate significantly different values (p < 0.05) from the control.

I activity. Protoplasts cultured on NAA and BA containing media showed insignificant deviation from the control values. Thus, induction of glyoxalase I was specific to NAA treatment alone.

4.4 Effect of short term treatment of growth regulators on protoplast culture

After the extraction, protoplasts were cultured on CPW13M medium supplemented with
### Table 3
Effect of hormones on activity of glyoxalase I (I.U.) during *Nicotiana* protoplast culture.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>NAA</th>
<th>BA</th>
<th>NAA+BA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.029</td>
<td>0.29 (100)</td>
<td>0.029 (100)</td>
<td>0.029 (100)</td>
</tr>
<tr>
<td>4</td>
<td>0.030</td>
<td>0.032 (103.9)</td>
<td>0.030 (100)</td>
<td>0.032 (103.9)</td>
</tr>
<tr>
<td>8</td>
<td>0.034</td>
<td>0.034 (100)</td>
<td>0.032 (94.1)</td>
<td>0.034 (100)</td>
</tr>
<tr>
<td>12</td>
<td>0.036</td>
<td>0.038 (105.5)</td>
<td>0.033 (91.6)</td>
<td>0.037 (103.9)</td>
</tr>
<tr>
<td>16</td>
<td>0.048</td>
<td>0.064 * (133.3)</td>
<td>0.045 (93.75)</td>
<td>0.052 (108.3)</td>
</tr>
<tr>
<td>20</td>
<td>0.044</td>
<td>0.053 * (120.4)</td>
<td>0.039 * (84.1)</td>
<td>0.042 (95.4)</td>
</tr>
<tr>
<td>24</td>
<td>0.041</td>
<td>0.047 * (114.6)</td>
<td>0.038 (92.69)</td>
<td>0.038 (92.7)</td>
</tr>
<tr>
<td>28</td>
<td>0.038</td>
<td>0.035 (92.1)</td>
<td>0.036 (94.74)</td>
<td>0.035 (92.1)</td>
</tr>
</tbody>
</table>

Values in parenthesis indicate % difference, taking the control value as 100.

* indicate significantly different values (p < 0.05) from the control.

Different sets of hormones: NAA, BA, kinetin, zeatin, NAA+BA at 1ppm concentration each and inhibitors: colchicine and Isoascorbate (1mM) for 5 min. No growth regulator was added in the control. After the treatment protoplasts were washed twice with culture medium, the cell number and glyoxalase I activity were studied 20 h after incubation. The results indicate that NAA increased both the enzyme activity (128%) and cell division (154%) after 20 h of culture (Table 4). BA or other cytokinins do not show such pronounced effect on cell division as compared to NAA, but a slight increase in cell number over the control was observed. None of the cytokinins increased glyoxalase activity.
Table 4  Effect of various growth regulators on glyoxalase I activity and cell number

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>Enzyme act. (I.U.)</th>
<th>% change in Enz. act.</th>
<th>Cell No. 10^6/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>0.136±0.002</td>
<td>100</td>
<td>45±0.87</td>
</tr>
<tr>
<td>2.</td>
<td>NAA (1ppm)</td>
<td>0.174±0.025</td>
<td>128</td>
<td>70±1.44</td>
</tr>
<tr>
<td>3.</td>
<td>BA (1ppm)</td>
<td>0.106±0.036</td>
<td>77.9</td>
<td>55±1.50</td>
</tr>
<tr>
<td>4.</td>
<td>NAA+BA (1ppm each)</td>
<td>0.131±0.004</td>
<td>96</td>
<td>60±1.20</td>
</tr>
<tr>
<td>5.</td>
<td>D-Isoascorbate (1mM)</td>
<td>0.038±0.009</td>
<td>29</td>
<td>16±1.60</td>
</tr>
<tr>
<td>6.</td>
<td>Colchicine</td>
<td>0.075±0.005</td>
<td>55</td>
<td>30±1.10</td>
</tr>
<tr>
<td>7.</td>
<td>Zeatin (4.4uM)</td>
<td>0.122±0.002</td>
<td>89</td>
<td>56±1.20</td>
</tr>
<tr>
<td>8.</td>
<td>Kinetin (4.4uM)</td>
<td>0.128±0.005</td>
<td>94</td>
<td>59±1.15</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E of atleast three experiments.

activity over the control. In the set containing both NAA and BA, the effect is not independent of either treatments but the cell number and glyoxalase activity are in between NAA or BA alone Thus, the effect of auxin and cytokinin combination is a vectorial sum of cell number and enzyme activity.
5.1 Cell cycle analysis of mesophyll protoplast of tobacco

The growth of plant cells in culture, particularly the growth of single cells or small aggregates of cells in liquid medium of defined composition provides the ideal environment to study control of cell division cycle. Large homogenous population of cells can be subjected to controlled alterations in nutrient and growth factor concentrations and easily subsampled to provide independent estimates of cell number doubling time and various parameters controlling cell cycle phase (Bayliss, 1985).

With the sophistication of techniques and availability of improved technology, the picture of cell cycle control is becoming clearer (Toonan et al. 1996). One of such techniques is flow cytometry (Darzynkiewics, 1994). Protoplasts are favourable materials for cell sorting as they lack the cell wall and are uniformly spherical in shape.

Protoplasts are usually isolated from leaf mesophyll cells simply because these provide a convenient source of large number of identical cells. Galbraith et al. (1981) used microfluorimetric measurements of nuclear DNA content on freshly isolated protoplasts to show a single peak of DNA content. He concluded that mesophyll cells are non-meristematic and are probably arrested at G1. Unfortunately, he found no recognizable mitotic stages in diploid cells to fix the G1 and G2 DNA contents. Therefore, he assumed that protoplast isolated from mature mesophyll cells were arrested at G1.

The output histograms from the flow cytometer indicate two peaks. In order to identify the peak for 4C nuclei, colchicine treated protoplasts were used. The second peak was found to corresponds to colchicine arrested cells, thus confirming the location of 4C nuclei on the X axis (DNA content). In the present investigation, we found that majority of the freshly isolated
protoplasts were located in $G_1/G_0$ phase of cell cycle. During the monitoring of cell cycle, a very low (0.0 - 0.9) percent cells were found to be in $G_2/M$ in the initial period but, between 18-30 h of culture, there is a sudden increase in the cells entering $G_2/M$. The transition from $G_1$ to $S$ to $G_2/M$ was short and occurred only in 30% of the total cell population. These results suggested that the actively dividing cells show 30% synchrony.

Synchronization of cultures by limiting growth factors is unlikely to cause cells to arrest in $G_2$ phase of the cell cycle (Nishi et al. 1977). Thus, a homogenous and fully synchronised culture was impossible to achieve by artificially manipulating growth conditions. Catharanthus roseus (Amino et al. 1983) and Helianthus tuberosus (Serafini-Fracassini et al. 1980) provide interesting materials showing 70-90% synchronised population of cells. We found that inspite of poor cell division activity in freshly isolated protoplasts (as no growth regulators were provided to induce cell division in non dividing cells) a small subpopulation did enter into $G_2$-$M$ phase between 18 to 24 h and by 30 h first cell cycle was completed. Culture of isolated protoplasts in the presence of auxin and cytokinin leads apparently to semi-synchronous progression through $S, G_2$ and $M$ (Cooke and Meyer, 1981).

The peak in cells entering $G_2/M$ follows immediately after the cells show a peak in glyoxalase I activity. Thus, glyoxalase I activation occurs just prior to $G_2/M$ phase and has a definite role in cell division cycle

5.2 Cell division and glyoxalase I activity.

Although a number of reports confirm correlation of glyoxalase I activity with rate of cell division (Ramaswamy et al. 1983; Das et al. 1987; Sethi et al. 1988), the exact mechanism of involvement of glyoxalase I in cell division is not yet known.

On monitoring cell number and glyoxalase I activity in tobacco mesophyll protoplasts we find that cell division shows an exponential increase between (18-24) h whereas the glyoxalase I activity peaks at 18 h i.e. just before the cells start dividing. The instantaneous increase in cell
number after protoplast isolation or the incubation of cells arrested at $G_1$ (as explained by Galbraith, 1981) were initiated to re-enter into cell cycle can be due to wound response (Simard, 1971). This data suggests that activation of glyoxalase I is required prior to cell division. On similar lines (Paulus et al. 1993) reported that when auxin starved soybean suspension cultures were re-initiated to divide, the glyoxalase I activity peaks one day before the rapid cell division. Then onwards, the glyoxalase I and cell division curves go parallel to each other. Thus, confirming the correlation of glyoxalase I with cell division.

5.3 Effect of hormones on protoplast division and glyoxalase I activity

It has been reported that auxin and cytokinin are an absolute requirement for the growth of protoplasts (Takahashi, 1995). A number of reports confirm auxin induced progression of arrested cells to DNA synthesis phase e.g. in carrot cell suspensions (Nishi et al. 1977) Acer cultures (Everett et al. 1981), Soybean cell suspension (Paulus et al. 1993) and tobacco cell suspension culture (Droog et al. 1993). However, when cytokinin starved suspension cultures of tobacco were resupplied with cytokinin, no effect on DNA synthesis or cell number was observed (Joanneau & Tandeau de Marsac, 1973). Cytokinin deprivation from the medium reduced mitotic index and cells became endopolyploid. Thus, cytokinin involvement with a putative $G_2$ control point seems to be associated with switch between normal and amitotic cell cycles rather than an absolute requirement for cell cycle progression (Bayliss, 1985).

The classical experiment by Skoog and Miller (1957) established that phytohormones interact synergistically to produce the response i.e. cytokinins act together with auxins to stimulate cell divisions in tobacco.

We find that when auxin and cytokinin are supplemented in protoplast growth medium, an increase (20%) in cell number is seen after 28 h of culture. But auxin alone was effective in increasing the cell number to 30% whereas, in cytokinin medium alone can increase was 10%. The glyoxalase I activity shows that there is a definite and specific percentage of enzyme induction by auxin whereas cytokinin could not induce glyoxalase I. Auxin induction of
glyoxalase I has also been reported by Paulus et al. (1993). The use of specific inhibitors of auxin (e.g. TIBA) could further confirm these results (Hobbie et al. 1994).

5.4 **Effect of short term treatment of hormones and inhibitors on protoplast growth**

Short term treatments were conducted to find out whether continuous treatments of hormones could be reproduced in cell division with brief 5 min pulse treatments on cell division and enzyme activity.

We find that long term treatments support better growth. Since the treatment procedure was same for all sets i.e. after isolation, protoplasts were incubated in the treatment solution for 5 min. and then washed twice with CPW21S along with centrifugation, the experimental hazards reduced the values of cell number and enzyme activity in control tissues too. The relative effect of hormones and inhibitors was compared with the control where, CPW21S media was added for 5min. More or less, similar trend was observed i.e. auxin induced cell division and glyoxalase I activity maximum as compared to cytokinin or NAA+BA set. BA lowered the enzyme activity by 22% whereas, kinetin showed 6% and zeatin showed 11% decrease. The cell cycle inhibitor, colchicine reduced glyoxalase I activity by 45% and glyoxalase I specific inhibitor. Isoascorbate (Gray and Norton, 1980) could reduce the cell number (approx. 30%).

This proves conclusively that glyoxalase I and cell number are intimately connected.

It remains to be seen what relation these phytohormones have with cell cycle studies. Is there any stage specific effect or the requirement of phytohormones is throughout the stages of cell cycle, what are the factors coordinating these signal response function and at what stage does glyoxalase I fits in.
SUMMARY AND CONCLUSIONS

Plant growth and morphogenesis are the products of localized cell division and anisotropic cell expansion. Thus, the cellular mechanisms of development can be better understood only when the control of cell proliferation is deciphered. In this part of the dissertation, investigations were carried out to understand the cell cycle controls in *Nicotiana* protoplasts. The results obtained are as follows:

1). The flowcytometric analysis of protoplasts monitored for 30 h after isolation showed that freshly isolated protoplasts contained 52.5% cells at G₁/G₀ phase of cell cycle. Most of the cells passed through G₂/M phase after 24 h of culturing. Total time for the first cell cycle was ca. 30 h.

2). Glyoxalase I activity was found to increase just prior to increase in cell number.

3). Auxin was found to increase the rate of cell division as well as glyoxalase I activity in protoplast cultures whereas, cytokinin did not show any significant change. Auxin and cytokinin combination was found to exhibit a synergistic effect.

4). Short term treatment of phytohormones and specific inhibitors of cell division and glyoxalase I for 5 min followed by culturing of protoplasts for 24 h showed that NAA was most effective to enhance cell number as well as enzyme activity whereas, cytokinins (BA, zeatin and kinetin) showed marginally lower values. An inhibitor of glyoxalase I (Isoascorbate) reduced cell number remarkably, and the cell cycle blocker - colchicine inhibited enzyme activity.

Thus, the present studies indicate that in tobacco protoplast cultures, glyoxalase I activity increased just prior to rapid cell division. Glyoxalase I was found to be specifically induced by auxin even by a short treatment (5 min). The same result could not be achieved with BA, zeatin and kinetin - the known cell division hormones.
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