Chapter 4

Cell Culture

4.1. INTRODUCTION

At the turn of the 19th century Haberlandt (1902) made pioneering attempts to isolate and culture single cells from the leaves of flowering plants. He had envisaged that such a system would provide an excellent opportunity for investigating the properties and potential of plant cells and also to understand the interrelationships and complementary influences of cells in multicellular organisms (see Krikorian and Berquam, 1969). Although Haberlandt failed to achieve the division of free cells for various reasons (see Chapter 1), his detailed paper of 1902 stimulated several workers to pursue this line of investigation. To date the progress in this field has been so spectacular that it is possible not only to culture free cells but also to induce divisions in a cell cultured in complete isolation and to raise whole plant from it.

Plant physiologists and plant biochemists have recognized the merits of a single cell system over intact organs and whole plants for studying cell metabolism and the effects of various substances on cellular responses. The free cell system permits quick administration and withdrawal of diverse chemicals and radioactive substances (Gnanam and Kulandaivelu, 1969; Edwards and Black, 1971; Harada et al., 1972). The cloning of single cells permits crop improvement through the extension of the techniques of microbial genetics to higher plants. Large scale cultivation of plant cells in vitro provides a viable alternative for the production of vast arrays of commercially important phytochemicals (Chapter 17).

4.2. ISOLATION OF SINGLE CELLS

4.2.1. From intact plant organs

(i) *Mechanical method.* Leaf tissue is the most suitable material for the isolation of single cells. Ball and Joshi (1965), Joshi and Noggle (1967), and Joshi and Ball (1968) isolated cells from mature leaves of *Arachis hypogaea* by first tearing across the leaf to expose the mesophyll cells, followed by scraping of the cells with a fine scalpel. The isolated cells

were directly placed into liquid medium (for composition see Table 4.2). Many of the free cells were viable and underwent sustained divisions in culture. This is the first report demonstrating that free cells capable of dividing in artificial medium can be isolated from intact plant organs. However, these workers were unable to isolate viable cells from the leaves of most other plants they tested.

Gentle grinding of the leaves followed by cleaning the cells by filtration and centrifugation is now widely used for the isolation of mesophyll cells. Gnanam and Kulandaivelu (1969) isolated mesophyll cells active in photosynthesis and respiration from mature leaves of several species. The procedure involved mild grinding of 10 g leaves in 40 ml of the grinding medium (20 μmol sucrose, 10 μmol $MgCl_2$, 20 μmol Tris–HCl buffer, pH 7.8) with a mortar and pestle. After filtering the homogenate through two layers of fine muslin cloth the released cells were washed by low centrifugation in the grinding medium. Besides dicots, many monocots, including grasses (unidentified), yielded intact mesophyll cells by this procedure. Edwards and Black (1971) used a similar method to isolate metabolically active mesophyll cells and bundle sheath cells from crabgrass (*Digitaria sanguinalis*), and mesophyll cells of spinach.

Rossini (1969, 1972) described a method for the large-scale mechanical isolation of free parenchymatous cells from the leaves of *Calystegia sepium*. This method was later used successfully with *Asparagus officinalis* and *Ipomoea hederifolia* by Harada et al. (1972). The details of the procedure followed by these workers are given in Appendix 4.I.

Mechanical isolation of cells has at least two distinct advantages over the enzymatic method: (a) it eliminates the exposure of cells to the harmful effect(s) of enzymes, and (b) in this method the cells need not be plasmolyzed which is often desirable in physiological and biochemical studies. Schwenk (1980) isolated viable cells from soybean cotyledons in sterile distilled water. In several cases the mechanically isolated cells have been reported to divide and form callus (Ball and Joshi; 1965; Kohlenbach, 1966; Rossini, 1972; Schwenk, 1980).

Although Gnanam and Kulandaivelu (1969) were able to isolate cells from the leaves of several species, the mechanical method of cell isolation is not universally applicable. Mesophyll cells could be successfully isolated by this method only in such cases where the parenchymatous tissue was loosely arranged, having few points of cell to cell contact (Rossini, 1972).

(ii) *Enzymatic method*. Enzymatic isolation of single cells has been practised for quite some time by plant physiologists and biochemists (Chayen, 1952; Zaitlin, 1959; Sato, 1968). A procedure for the large-scale

isolation of metabolically active mesophyll cells of tobacco by pectinase treatment was first reported by Takebe et al. (1968) and later extended by Otsuki and Takebe (1969) to 18 other herbaceous species. For the details of their method, see Appendix 4.II.

Takebe et al. (1968) demonstrated that the presence of potassium dextrane sulphate in the maceration mixture improved the yield of free cells. The enzyme macerozyme used to isolate cells not only degrades the middle lamella but also weakens the cell wall. It is, therefore, essential that in the enzymatic method of cell isolation the cells are provided with osmotic protection. Tobacco protoplasts collapsed within the cell wall when mannitol was used at a concentration below 0.3 M (Takebe et al., 1968).

A special feature of enzymatic isolation of cells is that in some cases it has been possible to obtain pure preparations of spongy parenchyma and palisade parenchyma (Takebe et al., 1968). However, some plant species, especially *Hordeum vulgare*, *Triticum vulgare* and *Zea mays*, have proved difficult materials for cell isolation through the enzymatic methods (Zaitlin, 1959; Otsuki and Takebe, 1969). According to Evans and Cocking (1975) the mesophyll cells in these cereals appear elongated with a number of constrictions. Within the leaf these cells may form an interlocking structure preventing their isolation.

Rubos (1985) described a method to isolate viable single cells from zygotic embryos of cabbage, carrot and lettuce. The excised embryos were treated with 1% macerozyme, at 32°C, for 2 h, on a shaker (50 rev min^{-1}). The treated embryos were forced through a 5 ml hypodermic syringe several times to release single cells from the confines of the surrounding cuticular layer.

4.2.2. From cultured tissues

Traditionally, the single cell systems used in basic and applied research are isolated from cultured tissues because this approach is convenient and widely applicable. Cultures are initiated by simply placing freshly cut sections from surface-sterilized plant organs on a nutrient medium containing suitable hormones; generally auxins and cytokinins are used. On such a medium the explant exhibits callusing which usually starts at the cut ends and gradually extends over the entire surface of the tissue. The callus is separated from the parent explant and transferred to a fresh medium of the same composition to build up a reasonable amount of tissue. Repeated subculture on the agar medium may also improve the friability of the tissue which is highly desirable for raising a fine cell suspension in liquid medium (Wilson and Street, 1975; Noguchi et al., 1977). Wilson and Street (1975) observed that when freshly isolated callus of

Hevea brasiliensis was transferred to a liquid medium and agitated, it broke up into small pieces but all efforts to raise a fine suspension of cells from it failed. They transferred the callus pieces grown in liquid medium back to the agar medium. After 2 months a very friable callus was formed which on transfer to the liquid medium gave a fine suspension.

To obtain free cells, pieces of undifferentiated and friable calli are transferred to liquid medium in flasks or some other suitable vial and the medium is continuously agitated by a suitable device. Such cultures are called 'suspension cultures'. Agitation of the medium serves at least two functions. First, it exerts a mild pressure on cell aggregates, breaking them into smaller clumps and single cells and, secondly, agitation maintains uniform distribution of cells and cell clumps in the medium. Movement of the medium also provides gaseous exchange between the culture medium and culture air.

4.3. SUSPENSION CULTURES

4.3.1. General techniques

Basically there are two types of suspension cultures: batch cultures and continuous cultures.

(i) *Batch cultures.* These are used for initiating single cell cultures. Cell suspensions are grown in 100–250 ml flasks each containing 20–75 ml of culture medium. The cultures are continuously propagated by routinely taking a small aliquot of the suspension and transferring it to a fresh medium (ca. 5× dilution).

During the incubation period the biomass of the suspension cultures increases due to cell division and cell enlargement. This continues for a limited period after which the growth stops due to the exhaustion of some factors or the accumulation of certain toxic metabolites in the culture medium. If at this stage a small aliquot of the cell suspension, with uniformly dispersed cells and cell aggregates, is transferred to a fresh medium of the same composition (subculture), the cell growth is revived. The biomass growth in batch cultures follows a fixed pattern as shown in Fig. 4.1. Initially, the culture passes through a lag phase, followed by a brief exponential growth phase during which active cell divisions occur. After three to four cell generations the growth declines and finally the culture enters the stationary phase. The duration of the lag phase would largely depend on the growth phase of the stock culture at the time of subculture and the size of the inoculum (Stuart and Street, 1969). Cultures can be maintained continuously in exponential phase by frequent
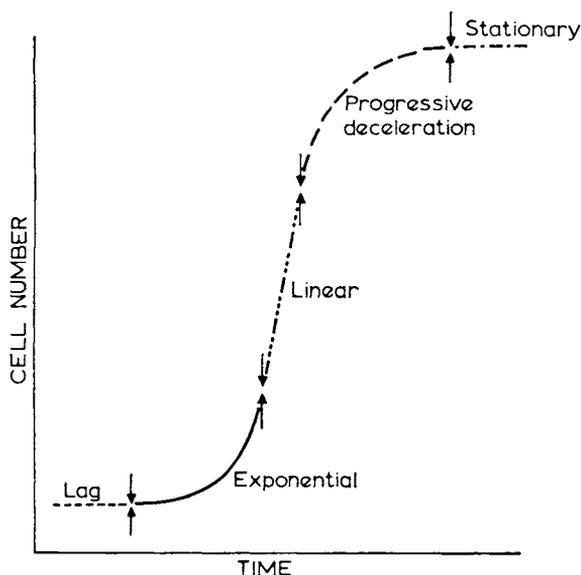
Fig. 4.1. Model curve relating cell number per unit volume of culture to time in a batch-grown cell suspension culture. Growth phases are labelled (after Wilson et al., 1971).

(every 2–3 days) subculture of the suspensions. Prolonged maintenance of cultures in the stationary phase may result in extensive death and lysis of cells. It is, therefore, critical that suspensions are subcultured soon after they have reached their maximum dry-weight yield (Street, 1977b). Addition of conditioned medium (in which cell cultures have been grown before) reduces the lag phase dramatically (Bergmann, 1977). Cell doubling time in suspension cultures varies with the tissue: *Nicotiana tabacum*, 48 h; *Acer pseudoplatanus*, 40 h; *Rosa* sp. 36 h; *Haplopappus gracilis*, 22 h (Butcher and Ingram, 1976).

For subculture of the suspension cultures a pipette or syringe with an orifice fine enough to allow single cells and small aggregates (2–4 cells) to pass through, but excluding larger cell clumps, is used. At the time of subculture the flask is allowed to stand for a few seconds to allow the large colonies to settle down, and suspension is taken from the upper part of the culture. Regular practice of this procedure should allow the build-up of a fine suspension.

The texture of a callus is genetically controlled and often it may be difficult to obtain a good dispersion of cells under any conditions. Generally, however, it has been possible to improve the tissue dissociation by manipulating the media composition and subculture routine. Addition of 2,4-D (Negrutiu and Jacobs, 1977), small amounts of hydrolytic enzymes

such as cellulase and pectinase (Street, 1977b), or substances like yeast extract (Noguchi et al., 1977), had a promotory effect on cell dispersion. Negrutiu and Jacobs (1977) reported that maximum dissociation of cells was achieved by permanently maintaining the cultures in the late log phase by adding fresh medium every other day in a proportion that the biomass/medium volume was kept at 2. To obtain a fine suspension culture it is of prime importance that, as far as possible, a friable callus is used initially. As mentioned earlier, friability of the callus tissue often increases if it is maintained on a semi-solid medium for 2–3 passages. However, it must be borne in mind that even the finest cell suspensions carry cell aggregates and no suspension culture is comprised exclusively of free cells.

Owing to certain inherent drawbacks of the system, batch cultures are not ideal for studies of cell growth and metabolism (Fowler, 1977; Street, 1977b; Wilson, 1980). Batch cultures are characterized by a constant change in the pattern of cell growth and metabolism, and the composition of the nutrient medium. In these cultures the exponential growth with a constancy of cell doubling time may be achieved for a short time but there is no period of steady-state growth in which the relative cell concentrations of metabolites and enzymes are constant (Wilson, 1980). To a certain extent these problems are overcome by continuous cultures.

(ii) *Continuous cultures*. A number of culture vessels have been designed to grow large-scale cultures under steady state for long periods by adding fresh medium and draining out the used medium (Street, 1977b). Continuous cultures may be of the close type or the open type. In the former, the addition of fresh medium is balanced by outflow of the old medium. The cells from the outflowing medium are separated mechanically and added back to the culture. Thus, in 'close continuous cultures' cell biomass continues to increase as the growth proceeds. In contrast, in the 'open continuous cultures' the inflow of the medium is accompanied by a balancing harvest of an equal volume of the culture (medium and cells). The rate of inflow of medium and culture harvest are so adjusted that the cultures are maintained at a constant, submaximal growth rate indefinitely (Fowler, 1977; Street, 1977b). Two major types of open continuous culture have been used: chemostat and turbidostat. In chemostat cultures, a steady state of cell growth is maintained by a constant inflow of the fresh medium in which the concentration of a chosen nutrient (nitrogen, phosphorus or glucose) has been adjusted so as to be growth-limiting. In such a medium all constituents other than the growth-limiting nutrient are present at concentrations higher than that required to maintain the desired rate of cell growth. The level of the growth-

TABLE 4.1

Culture medium for suspension cultures of tobacco[a]

Constituents	Amounts (mg l^{-1})
Inorganic salts	As in MS medium
Thiamine·HCl	10
Pyridoxine·HCl	10
Nicotinic acid	5
myo-Inositol	100
Casein hydrolysate	1000
2,4-D	2
Kinetin	0.1
Sucrose	30000
pH	5.7

[a]After Reynolds and Murashige (1979).

limiting factor is so adjusted that any increase or decrease in it is reflected by corresponding increase or decrease in the growth rate of cells. In turbidostat cultures, the input of fresh medium is intermittent, controlled by an increase in the turbidity of the culture from cell growth. A preselected biomass density is maintained by the wash-out of cells.

Chemostat cultures offer the possibility of maintaining a steady-state of cell growth and metabolism, and to determine the effect of individual growth-limiting nutrients on cell growth. However, despite the clear-cut advantages, continuous cultures are not used widely for large scale plant cell culture, probably because these cultures require a lot of attention, and the equipment is not generally available (Wilson, 1980).

4.3.2. Culture medium for suspensions

The medium used for raising fast growing friable callus should generally prove suitable for initiating suspension cultures of that species provided, of course, agar is omitted from it. Manipulation of the auxin/cytokinin ratio to achieve better cell dispersion is desirable. For tobacco, increasing the concentration of 2,4-D from 0.3 mg l^{-1} to 2 mg l^{-1} and supplementing the callus medium with additional vitamins and casein hydrolysate (see Table 4.1) have been recommended (Reynolds and Murashige, 1979).

In actively growing suspension cultures the inorganic phosphate is rapidly utilized and, consequently, it soon becomes a limiting factor (Eriksson, 1965; Noguchi et al., 1977). Noguchi et al. (1977) have demonstrated that in tobacco suspension cultures maintained in a medium with

standard MS salts the phosphate concentration declines to almost zero within 3 days of the initiation of culture. When the phosphate concentration in the medium was raised three times the original level, it was completely utilized by the cells within 5 days. B_5 and ER media given in Table 3.1 were developed for suspension cultures of higher plants. These and other synthetic media are normally suitable only if the initial population density is around 5×10^4 cells ml^{-1} or higher. With a lower cell density the medium needs to be enriched with various other components (see Section 4.4.2).

4.3.3. Agitation of the medium

To achieve movement of the culture medium in suspension cultures various types of set-up have been used. Muir (1953), who for the first time demonstrated that cells of tobacco and *Tagetes erecta* can be cultured in suspension cultures, used an orbital platform shaker. This is still the most popular method of growing batch suspension cultures. The platform of the shaker is fitted with clips of appropriate size for holding the flasks. Often the clips are interchangeable to permit the use of flasks of different sizes. An orbital shaker with a variable speed of 30–150 rev. min^{-1} is satisfactory (speed above 150 rev. min^{-1} is unsuitable; Street, 1977b) with stroke in the range of 2–3 cm orbital motion.

4.3.4. Synchronization

A synchronous culture is one in which the majority of cells proceed through each cell cycle phase (G_1, S, G_2 and M) simultaneously. The degree of synchrony is expressed as percentage synchrony.

For studying cell cycle and cell metabolism in suspension cultures it is advantageous to use synchronous or partially synchronous cultures, which exhibit amplification of each event of the cell cycle as compared to non-synchronous cultures (King, 1980). Since cell suspension cultures are normally asynchronous, considerable efforts have been made to achieve a reasonable synchrony. Synchronization of asynchronous cultures is characterized by sequential alternation in frequency distribution of different cell cycle events. It has been emphasized by King and Street (1977) and King (1980) that the degree of synchrony should not be determined solely by mitotic index; it should be based on a number of parameters determined independently, e.g. (a) the percentage of cells at a specific point in the cycle at one moment in time, (b) the percentage of cells passing a specific point in the cycle during a brief specific period, and (c) the percentage of total time required for all the cells to pass a specific point in

the cycle. The methods used to achieve synchronization of cell suspensions fall under two categories: starvation and inhibition.

(i) *Starvation.* In this method cells are first arrested in the G_1 or G_2 phase of the cell cycle by starving them of a nutrient or hormonal factor required for cell division. After a period of starvation when the limiting factor is supplied into the medium the stationary cells enter division synchronously.

Up to 80–90% of the cells in the explants of tuber tissue of *Helianthus tuberosus* excised in low intensity green light and cultured in the dark on a nutrient medium containing 2,4-D divided synchronously (Mitchell, 1967; Yeoman and Evans, 1967; Davidson and Yeoman, 1974; Fraser and Loening, 1974; Aitchison et al., 1977). Suspension cultures of *Acer pseudoplatanus* from the stationary phase entered synchronous division when they were inoculated into fresh medium at low density (Street, 1968). The cells in the stationary phase are locked up in the G_1 phase of the cell cycle (Bayliss and Gould, 1974; King and Street, 1977), which is probably due to the depletion of nitrate ions in the medium (King, 1977). In large-scale cell cultures of *A. pseudoplatanus* a high level of cell synchrony was maintained over five cell cycles, as revealed by the step-wise increase in cell number at each successive cytokinesis. Komamine et al. (1978) achieved synchrony in *Vinca rosea* cultures by starving them of phosphate for 4 days and then transferring to phosphate-containing medium.

Growth hormone starvation of cells has been used to synchronize cell cultures of *Nicotiana tabacum* var. Wisconsin 38 (cytokinin; Jouanneau, 1971; Peaud-Lenoel and Jouanneau, 1971) and *Daucus carota* (auxin; Nishi et al., 1977).

(ii) *Inhibition.* Inhibitors of DNA synthesis, such as 5-aminouracil (Eriksson, 1966; Mattingley, 1966; Kovacs and Van't Hof, 1970; Butenko et al., 1974), FUdR (Blaschke et al., 1978; Cress et al., 1978), hydroxyurea and thymidine (Eriksson, 1966), have been used to synchronize cell cultures. When cells are treated with these chemicals the cell cycle proceeds only up to the G_1 phase and the cells are collected at the G_1/S boundary. Removal of the inhibitor is followed by synchronous division of cells. In this method cell synchrony is limited to only one cell cycle (King, 1980). Periodic flushing of chemostat cultures of *Glycine max* with nitrogen (Clowes, 1976) or ethylene (Constabel et al., 1977) are also reported to induce cell synchrony. However, in all these cases the only evidence of synchrony produced was fluctuation in the mitotic index which is not entirely satisfactory (see King, 1980).

4.3.5. Assessment of growth in suspension cultures

Growth in plant cell suspension cultures is commonly measured by cell counting, determination of total cell volume (packed cell volume, PCV), and fresh and dry-weight increase of cells and cell colonies.

(i) *Cell counting*. Since suspension cultures invariably carry cell colonies of various sizes it is difficult to make a reliable counting of cell numbers by taking samples directly from the flask. The accuracy of cell counting may be improved if the cells and cell aggregates are first dispersed by treating them with chromic acid (5–8%) or pectinase (0.25%). The method followed by Street and co-workers (Street, 1977b) to count sycamore cells is as follows: add 1 volume of culture to 2 volumes of 8% chromic trioxide and heat to 70°C for 2–15 min (the duration is determined by the growth of the culture). Cool the mixture and shake vigorously for 10 min before counting the cells in a haemocytometer.

(ii) *Packed cell volume* (PCV). To determine PCV transfer a known volume of uniformly dispersed suspension to a 15-ml graduated centrifuge tube and spin at 200 $\times g$ for 5 min. PCV is expressed as ml pellet ml^{-1} culture.

(iii) *Cell fresh weight* (FW). This can be determined by collecting cells on a pre-weighed (in wet condition) circular filter of nylon fabric supported in a Hartley funnel, washing the cells with water to remove the medium, draining under vacuum, and reweighing.

(iv) *Cell dry weight* (DW). Follow as above using a pre-weighed dry nylon filter and after collecting the cells on the filter dry them for 12 h at 60°C and reweigh. Cell weight is expressed as per culture or per 10^6 cells.

(v) *Non-invasive methods*. All four methods described above require withdrawal of culture samples. Recently two non-invasive methods to characterize growth in batch cultures have been described.

Schripsema et al. (1990) observed a direct correlation between loss of weight of the contents of a culture vessel and the curve representing dissimilation of sugar into CO_2. The dissimilation curve clearly shows the different growth phases, viz. lag phase, exponential phase and stationary phase. Therefore, by periodic weighing of a culture flask, provided with a stable closure (e.g. Silicosen T-type plugs) for accurate correlation of water loss, a dissimilation curve can be prepared and the growth characterized.

In the method suggested by Blom et al. (1992), the culture flask, fitted with a ruler, is tilted at an angle of 30° or 60° (same each time) for 5 min and the height of the sediment recorded. The change in the height of the sediment with the age of the culture would represent the change in fresh weight of the cells as there is a direct correlation between the two parameters.

4.3.6. Assessment of viability of cultured cells

(i) *Phase contrast microscopy*. Microscopic assessment of cell viability is based on cytoplasmic streaming and the presence of a healthy nucleus (Negrutiu and Jacobs, 1977). While the phase contrast microscopy gives a better picture of these feature, it is often not difficult to observe them under bright field microscopy.

(ii) *Reduction of tetrazolium salts*. In this test the respiratory efficiency of cells is measured by reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the red dye formazan. Formazan can be extracted and measured spectrophotometrically. Although this method allows quantification of observations, it alone may not always give a reliable picture of the cell viability (Withers, 1980).

(iii) *Fluorescein diacetate* (FDA) method. This technique offers a quick visual assessment of percentage viability of cells. Stock solution of FDA at a concentration of 0.5% is prepared in acetone and stored at 0°C. To test viability it is added to the cell or protoplast suspension (for protoplasts, an appropriate osmotic stabilizer is added to the FDA solution) at a final concentration of 0.01%. After about 5 min incubation the cells are examined, using a mercury vapour lamp with a suitable excitation filter and suppression filter. FDA is non-fluorescing and non-polar, and freely permeates across the plasma membrane. Inside the living cell it is cleaved by esterase activity, releasing the fluorescent polar portion fluorescein. Since fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cells, but in dead and broken cells it is lost. When illuminated with UV light it gives green fluorescence (Widholm, 1972; Larkin, 1976).

(iv) *Evan's blue staining*. The stain can be used complementary to FDA. When the cells are treated with a dilute (0.025%) solution of Evan's blue the damaged cells take up the stain but the intact and viable cells exclude it and, thus, remain unstained.

4.4. CULTURE OF SINGLE CELLS

In his pioneering attempt to culture mechanically isolated mesophyll cells, Haberlandt (1902) was successful in maintaining the cells alive for about 10 days. During this period cell swelling and wall thickening occurred but the cells failed to divide. Schmucker (1929) reported that mechanically isolated cells from the leaves of *Macleaya cordata* divided repeatedly in filter-sterilized sap of the same leaves. Kohlenbach (1959, 1965) confirmed the ability of these cells to undergo sustained divisions. Since then steady progress has been made in this field of research.

Ball and Joshi (1965) used time-lapse photomicrography and studied the development of an individual mesophyll cell of peanut in liquid medium. They noted that after 3–5 days in culture the leaf cell increased in size such that it no longer resembled a palisade cell. Accumulation of plastids around the nucleus (systrophy) preceded actual cell division. According to these authors only palisade cells divided, whereas the spongy parenchyma cells died. Later, Jullien (1970) demonstrated that spongy cells of peanut are also capable of dividing provided the isolation of the cells has been carried out properly. Similarly, Rossini (1972), who made cinematographic studies of the cultures of mesophyll cells of *Calystegia*, observed that both palisade and spongy parenchyma cells undergo division (see Fig. 4.2). Under optimum conditions 60% of these cells divided. However, the division of spongy cells occurs slightly later than that of palisade cells.

4.4.1. Techniques of single cell culture

The most popular technique of single cell culture is Bergmann's cell plating technique (Bergmann, 1960; see Fig. 4.3). Free cells are suspended in a liquid medium at a density twice the finally desired plating cell density. Melted agar (0.6–1%)-containing medium of otherwise the same composition as the liquid medium is cooled and maintained at 35°C in a water bath. Equal volumes of the two media are mixed and rapidly spread out in petri dishes in such a manner that the cells are evenly distributed and fixed in a thin layer (ca. 1 mm thick) of the medium after it has cooled and solidified. The dishes are sealed with parafilm. Suspension cultures which carry cell aggregates in addition to free cells should be filtered through a sieve which would allow only single cells and small cell aggregates to pass through. The large cell aggregates are discarded and only the fine suspension is plated. The plates may be observed under an inverted microscope and single cells marked on the outside of the plate by a fine marker to ensure the isolation of pure single cell clones.
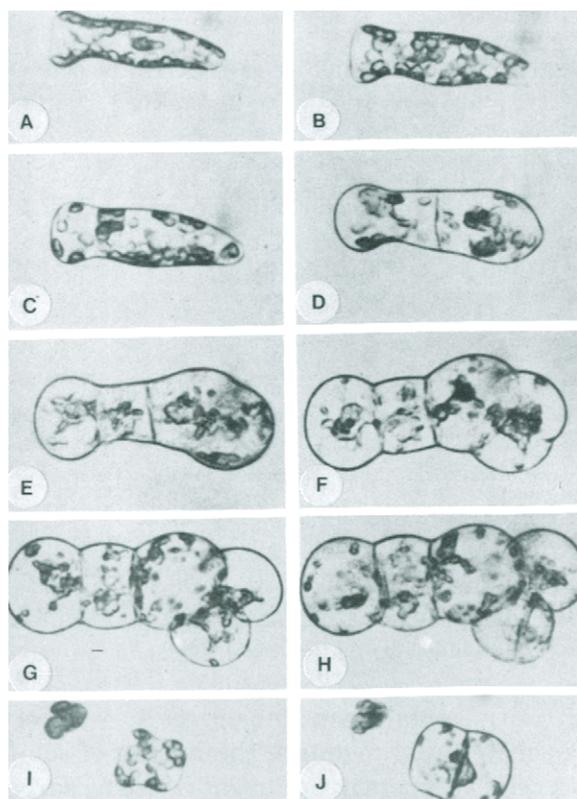
Fig. 4.2. Time-lapse pictures of divisions in an isolated palisade (A–H) and a spongy cell (I,J) of *Calystegia sepium.* A,I. Cells on the day of inoculation. B–H. 1,2,3,4,5,6 and 7 days after inoculation. J. On the fifth day of inoculation (after Rossini, 1972).

The culture plates are incubated in the dark at 25°C. It has been a common experience that frequent inspection of plates under the microscope light during the incubation period adversely affects the development of the colonies (Street, 1977b). In such cases it would be advisable to keep the observations to a bare minimum.

Free single cells can also be plated in a thin layer of liquid medium as commonly practised for protoplast culture (see Chapter 12). Cells isolated directly from plant organs have been frequently cultured in a liquid medium (Ball and Joshi, 1965; Kohlenbach, 1965; Rossini, 1969). A disadvantage in using a liquid medium is that the follow-up of an individual cell and its derivatives is extremely difficult because the cells are not in a fixed position.

If the plating cell density is determined at the time of culturing and a known volume of suspension is transferred to each plate it should be
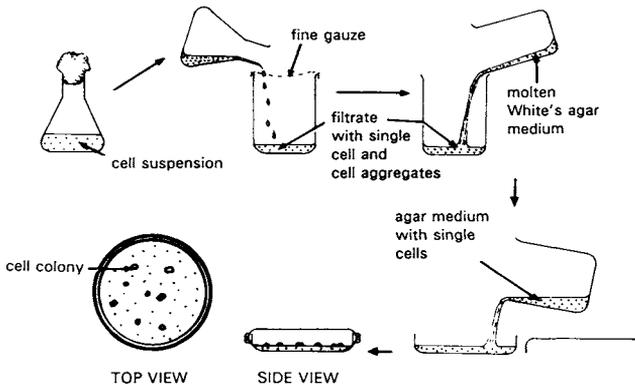
Fig. 4.3. Diagrammatic summary of steps involved in Bergmann's technique of cell plating (after Konar, 1966).

possible to make a quantitative assessment of plating efficiency using the formula:

$$\text{Plating efficiency} = \frac{\text{No. colonies / plate at the end of the experiment}}{\text{No. of cell units initially / plate}} \times 100$$

When cells are plated at an initial population density of 1×10^4 or 1×10^5 cells ml^{-1}, either in agar or in liquid medium, the mixing of colonies derived from neighbouring cells frequently occurs at a fairly early stage, much before they can be successfully diluted. This complicates the isolation of pure cell clones. The problem can be minimized if the effective plating cell density can be reduced or individual cells can be cultured in complete isolation. However, as in suspension cultures, under normal conditions there is a plating density optimum for each species, and cells fail to divide below a critical cell density. To grow single cells at low densities or individually, special requirements need to be fulfilled. Various methods have been described to grow individual cells.

(i) *The filter paper raft-nurse technique*. This method was developed by Muir et al. (1954) to culture single cells from suspension cultures and friable calli of tobacco and marigold. It involves cultivating individual cells on top of an actively growing callus separated by a piece of filter paper (see Fig. 4.4). In practice, individual cells are isolated from suspension cultures or a friable callus with the aid of a micropipette or microspatula. Several days before cell isolation, sterile 8×8 mm squares of filter paper are placed aseptically on top of the established callus of the same or different species. The filter paper is wetted by liquid and nutrients from the
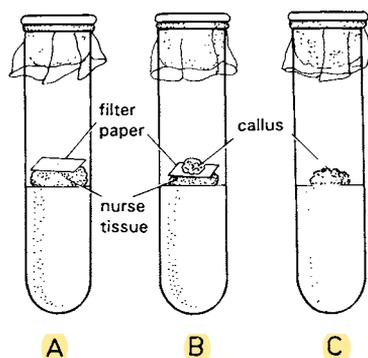
Fig. 4.4. Nurse-tissue technique to raise single cell clones. (A) A single cell from callus placed on filter paper lying on the top of a large callus (nurse tissue). (B) The cell has divided and formed a small tissue. (C) The tissue of single-cell origin has grown into a big callus after transfer from the filter paper to the medium directly (after Muir et al., 1958).

nurse tissue piece. The isolated single cell is placed on the wet filter paper raft. Cell transfer should be rapid in order to avoid excessive drying of the cell and the raft. After a macroscopic colony develops from the cell it is transferred to agar medium for further growth and maintenance of the cell clone.

An isolated cell which generally fails to divide when plated directly on the medium used for callus cultures is able to divide under the nursing effect of the callus. Apparently, the callus supplies the cell with not only the nutrients from the culture medium but something more that is critical for cell division. The cell division factor(s) can diffuse through the filter paper. The effect of callus tissue in stimulating the division of isolated cells can also be demonstrated by putting two callus pieces on an agar plate and seeding single cells around them. In such cultures cells close to the calli divide first. The beneficial effect of conditioned medium in single cell culture at low density is yet another evidence of the release of metabolites by growing tissues which are essential for cell division (see Section 4.4.2).

The feeder layer technique for low density cell plating used by some workers (Raveh et al., 1973; Vardi, 1978; Cella and Galun, 1980) is also based on the same principle as the raft-nurse technique (see Chapter 12).

(ii) *The microchamber technique*. This method was developed by Jones et al. (1960). They replaced the nurse tissue by a conditioned medium and grew single cells in a microchamber (see Fig. 4.5). In this method a drop of the medium carrying a single cell is isolated from suspension cultures, placed on a sterile microscope slide and ringed with sterile mineral
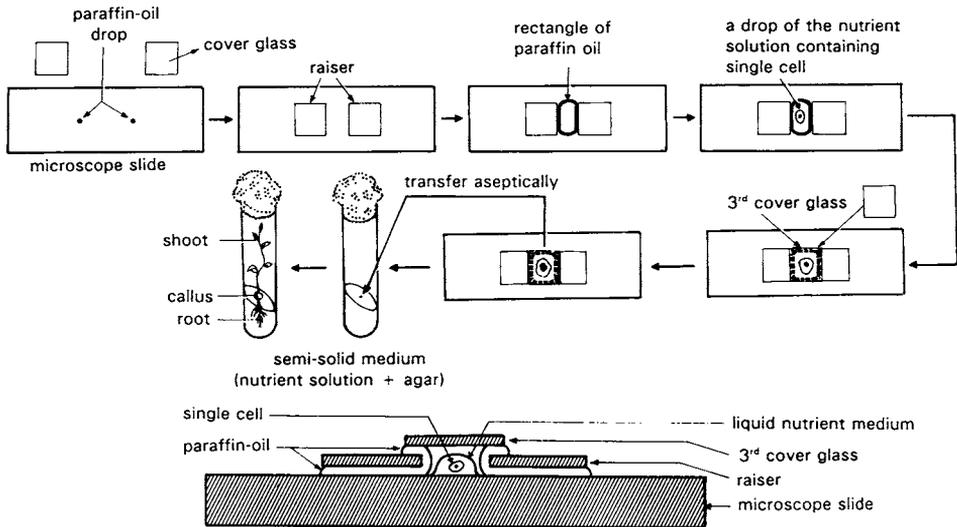
Fig. 4.5. Diagrammatic summary of the steps involved in the microchamber technique of cell cloning (after Jones et al., 1960).

oil. A drop of oil is placed on either side of the culture drop and a cover-glass placed on each drop. A third coverglass is then placed on the culture drop bridging the two coverglasses and forming a microchamber to enclose the single cell aseptically within the mineral oil. The oil prevents water loss from the chamber but permits gaseous exchange. The whole microchamber slide is placed in a petri-dish and incubated. When the cell colony becomes sufficiently large the coverglass is removed and the tissue is transferred to fresh liquid or semi-solid medium.

The microchamber technique permits regular observation of the growing and dividing cell. Vasil and Hildebrandt (1965) used the micro-chamber method and demonstrated that a complete flowering plant can be raised starting from an isolated single cell (see Fig. 4.6). Unlike Jones et al. (1960), they used a fresh medium containing mineral salts, sucrose, vitamins, Ca-pantothenate and coconut milk to culture a single cell.

(iii) *Microdrop method.* This method has been especially useful for culturing individual protoplasts but there is no reason why it cannot be equally effective for single cell culture (for details of this technique see Chapter 12).

4.4.2. Factors affecting single cell culture

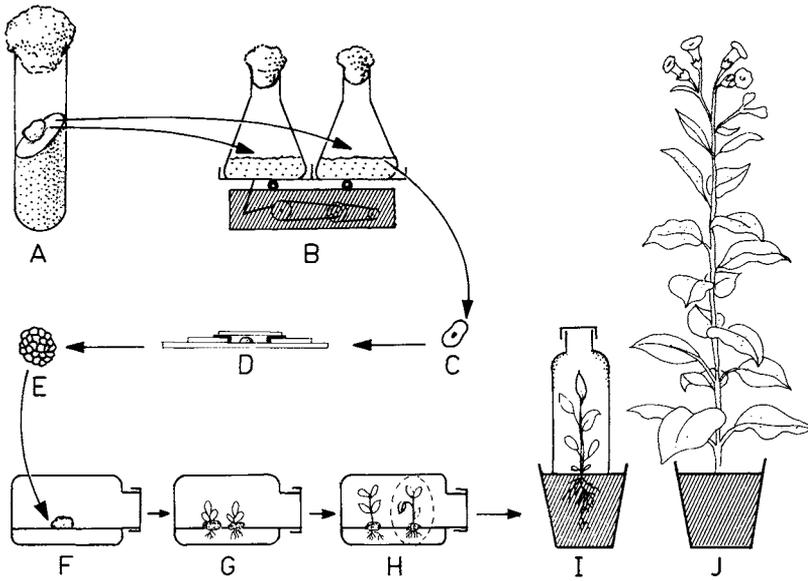The composition of the medium and the initial plating cell density are

Fig. 4.6. Development of a tobacco plant from a single cell. A callus is raised from a small piece of tissue excised from the pith (A). By transferring it to a liquid medium and shaking the culture flasks (B) the callus is dissociated into single cells. A cell (C) is mechanically removed from the flask and placed in a drop of culture medium in micro-chamber (D). A small tissue (E) derived from the cell through repeated divisions is then transferred to a semi-solid medium where it grows into a large callus (F), and eventually differentiates plants (G,H). When transferred to soil (I,J) these plants grow to maturity, flower and set seeds (from the work of Vasil and Hildebrandt, 1965).

critical for single cell culture. These two factors are interdependent. When cells are plated at a high density (5×10^4 or 1×10^5 cells ml^{-1}) a purely synthetic medium with a composition similar to that used for suspension cultures or callus cultures is generally satisfactory. The composition of three media employed for the culture of isolated mesophyll cells is given in Table 4.2.

The culture requirements of cells become increasingly complex as the plating cell density is decreased. This population effect on cell division can be effectively replaced by the addition to the minimal medium of undefined factors such as coconut milk, casein hydrolysate and yeast extract. Efforts have been made to develop synthetic culture medium for cells plated at low density. *Convolvulus* cells plated at low density required a cytokinin and amino acids that were not necessary for the callus culture of that species (Earle and Torrey, 1965a). Similarly, for sycamore cells plated at low density it was necessary to add a cytokinin, gibberellic acid and amino acids to the medium that was otherwise satisfactory for callus cultures of that plant (Stuart and Street, 1971). Kao and Michay

TABLE 4.2

Composition of media recommended for the culture of isolated mesophyll cells[a]

Constituents	Amounts (mg l^{-1})		
	Rossini (1972)	Joshi and Ball (1968)	Kohlenbach (1984)
KNO$_3$	950	–	950
KCl	–	750	–
NH$_4$NO$_3$	725	–	720
NaNO$_3$	–	600	–
MgSO$_4$·7H$_2$O	187	250	185
CaCl$_2$	169	–	–
CaCl$_2$·6H$_2$O	–	112	166
KH$_2$PO$_4$	69	–	68
NaH$_2$PO$_4$·2H$_2$O	–	141	–
NH$_4$Cl	–	5.35	–
MnSO$_4$·4H$_2$O	12.5	–	25
MnCl$_2$·4H$_2$O	–	0.036	–
H$_3$BO$_3$	5	0.056	10
ZnSO$_4$·4H$_2$O	5	–	10
ZnCl$_2$	–	0.15	–
NaMoO$_4$·2H$_2$O	0.125	0.025	0.25
CuSO$_4$·5H$_2$O	0.0125	–	0.025
CuCl$_2$·2H$_2$O	–	0.054	–
CoCl$_2$	–	0.02	–
FeSO$_4$·7H$_2$O	13.9	–	27.85
FeCl$_3$·6H$_2$O	–	0.5	–
Na·EDTA	18.6	–	37.25
Disodium salt of ethylene dinitrilotetraacetic acid	–	0.8	–
Adenine	–	–	20.25
Glutamine	–	–	14.7
Glycine	2	–	2
Nicotinic acid	5	–	5
Pyridoxine·HCl	0.5	–	0.5
Thiamine·HCl	0.5	–	0.5
Biotin	0.05	–	0.05
Folic acid	0.5	–	0.5
Casein hydrolysate (acid hydrolysate, acid and vitamin free)	–	400	–
myo-Inositol	100	–	100
BAP	0.1	–	–
Kinetin	–	0.1	1
2,4-D	1	1	1
Sucrose	10000	20000	10000

TABLE 4.2 (*continued*)

Constituents	Amounts (mg l^{-1})		
	Rossini (1972)	Joshi and Ball (1968)	Kohlenbach (1984)
pH	5.0	?	5.5

[a]Rossini (1972), for *Calystegia sepium*; Joshi and Ball (1968), for *Arachis hypogaea*; Kohlenbach (1984), for *Macleaya cordata, Zinnia elegans*, etc.

luk (1975) developed a rich but synthetic medium containing mineral salts, sucrose, glucose, a mixture of 14 vitamins, glutamine, alanine, glutamic acid and cysteine, a mixture of six nucleic acid bases, and a mixture of four organic acids of the TCA cycle, which supported division in cell cultures of *Vicia hajastana* at a density as low as 25–50 cells ml^{-1}. With the addition of casamino acids (250 mg l^{-1}) and coconut milk (20 ml l^{-1}) in place of the amino acids and nucleic acid bases in the above medium the effective plating cell density could be further reduced to 1–2 cells ml^{-1}. On a similar medium (for composition see Table 12.3) it was possible to culture individual protoplasts in separate dishes (each dish contained 4 ml of the medium). However, this medium proved ineffective for low density (80 or 800 protoplasts ml^{-1}) protoplast culture of *Solanum tuberosum* and *S. cardiophyllum*. Hunt and Helgeson (1989) succeeded in cultivating isolated single cells of these two species on a modified KM8p medium, in which sodium pyruvate, citric acid, malic acid and fumaric acid were omitted, the phosphate level was raised from 1.2 to 1.5 mM and 0.2% bovine serum albumin was added.

The cell density effect on cell division has been explained on the basis that cells synthesize certain compounds necessary for their division. The endogenous concentration of these compounds should reach a threshold value before a cell can embark on division. The cells continue to lose their metabolites into the medium until an equilibrium is reached between the cell and the medium. As a result, at high cell density the equilibrium is attained much earlier than at low density, and hence the lag phase is longer under the latter condition. Below a critical cell density the equilibrium is never reached and cells fail to divide. However, a conditioned medium which is rich in the essential metabolites is able to support divisions at a fairly low cell density. The inability to culture individual cells in a purely synthetic medium is due to our lack of adequate knowledge about the exact nature of the factor(s) responsible for cell division. A detailed analysis of conditioned medium should give some clues in this direction. Preliminary studies suggest that the conditioning factor/s are

TABLE 4.3

Comparison of the characteristics of microbial and plant cells

Characteristics	Typical microbial cell	Typical plant cell
Size (μm long)	2–10	50–100
Doubling time	1 h	2–6 days
Growth pattern	Single cell, pellets, mycelia	Clumps
Fermentation time	2–10 days	2–3 weeks
Oxygen requirement	1–3 mmol g^{-1} h^{-1}	10–100 mmol g^{-1} h^{-1}
Shear sensitivity	Insensitive	Sensitive
Water content (%)	Approx. 80	>90
Regulatory mechanism	Complex	Highly complex
Genetic makeup	Stable	May be highly variable
Product accumulation	Often extracellular	Mostly intracellular

After Panda et al. (1989) and Scragg (1991).

molecules which are stable at 25°C, non-volatile, acid and alkali tolerant and very polar with high molecular weight (700–1200 kDa), such as oligosaccharides and their derivatives (Bellincampi and Morpurgo, 1987; Birnberg et al., 1988; Schroder et al., 1989).

4.5. PLANT CELL REACTORS

Mass culture of plant cells in vitro has been proposed as a viable alternative for the production of vast arrays of high value, low volume phytochemicals (see Chapter 17). Therefore, during the past two decades considerable work has been done to design bioreactors for plant cell culture (Panda et al., 1989; Bisaria and Panda, 1991, Taticek et al., 1991; Scragg, 1991, 1994). A bioreactor is a glass or steel vessel in which organisms are cultured. Ideally, bioreactors are fitted with probes to monitor the pH, temperature and dissolved oxygen in the culture and have provisions to sample the cultures, add fresh medium, adjust pH, air supply, mixing of cultures and controlling the temperature, without endangering the aseptic nature of the culture. It, thus, allows closer control and monitoring of culture conditions than is possible using shake cultures.

Although the basic requirements for suspension cultures of plant cells are similar to those of submerged microbial cultures, the fermentors used for microbial cell cultures are not suitable for plant cell cultures because of striking differences in the nature and growth pattern of the two types of cells (Table 4.3).

Efficient mixing of plant cells cultured on large scale is extremely important to provide uniform physiological conditions inside the culture vessel. Mixing promotes better growth by enhancing the transfer of nutrients from liquid and gaseous phases to the cells and by break-off and dispersion of air bubbles for effective oxygenation. Although, plant cells have higher tensile strength in comparison to microbial cells, their large size, rigid cellulosic wall and extensive vacuole make them sensitive to the shear stress restricting the use of high agitation for efficient mixing. Plant cells are, therefore, often grown in modified stirred-tank bioreactors at very low agitation speeds. Air-lift reactors may provide even better and uniform environmental conditions at low shear.

All plant cells are aerobic and require continuous supply of oxygen. However, plant cells require less oxygen (1–3 mmol O_2 g^{-1} h^{-1}) than micro-organisms (10–100 mmol O_2 g^{-1} h^{-1}) because of their slow metabolism. In some cases, high oxygen concentration is even toxic to the metabolic activities of cells. Air is normally sparged or blown in at the base of the bioreactor.

Plant cells in suspension culture tend to form aggregates of 2–200 cells. During the late exponential phase of growth, cells become more sticky because of increased excretion of polysaccharides into the culture vessel. This leads to the adhesion of plant cells to the reactor wall, probes and stirring device and the formation of larger aggregates. Mixing is affected, as the aggregates tend to sediment or stick to the reactor surface, forming extensive wall growth. Large aggregates also create rheological problems by creating dead zones in the culture vessel and can block the opening and pipe lines of the reactor. Cell aggregation adversely affects the operation of the probes used to monitor culture conditions during growth and product formation. Diffusion-limited biochemical reactions may occur in large aggregates when nutrients can no longer penetrate to the aggregate's central core. In spite of these effects, certain degrees of cell aggregation (cell–cell contact) and cell differentiation seem to be essential for secondary metabolite production. Hence, controlled aggregation of plant cells is of interest from the process engineering point of view.

4.5.1. Selection of a bioreactor

The suitability of a particular bioreactor for plant cell cultivation could be evaluated by considering the following factors:
1. capacity of oxygen supply and intensity of air bubble dispersion in broth;

2. intensity of hydrodynamic stresses generated inside the reactor and their effect on the plant cell system;
3. adequacy of mixing of culture broth at high cell concentration;
4. ability to control temperature, pH, and nutrient concentration inside the reactor;
5. ability to control aggregate size (which may be helpful for increasing product formation);
6. ease of scale-up;
7. simplicity of aseptic operation for long durations.

4.5.2. Bioreactor designs

The large scale cultivation of plant cell suspension started in 1959 with NASA sponsored research on the possibility of using the cultures to supply food during space flight (Tulecke and Nickell, 1959, 1960). The vessels first used were large carboys and bottles which were either rolled or bubbled to give good mixing. These make-shift bioreactors were soon replaced by stainless steel bioreactors that are fitted with a motor and agitator. An air-lift bioreactor was introduced for plant cell culture in the

TABLE 4.4

The range of cell lines grown in bioreactors of different designs and capacities since 1959

Bioreactor system	Capacity (l)	Cell lines cultured	Period
Sparged carboy	3–10	*Ginkgo, Lolium, Mentha, Zea mays, Hyoscyamus niger*	1959–1975
Bubble-column	1.8–1500	*Glycine max Nicotiana tabacum*	1971–1975
Stirred-tank	2–15500	*N. tabacum, G. max, Petroselinum, Morinda citrifolia, Spinancia oleracea, Phaseolus vulgaris, Cudrania tricuspidata, Catharanthus roseus, Helianthus annuus, Coleus blumei*	1971– present
Air-lift	7–100	*M. citrifolia C. roseus, Theobroma, C. tricuspidata, Berberis wilsoneae, H. annuus, Cinchona ledgeriana*	1977– present
Rotating-drum		*C. roseus*	1983

After Scragg (1991).

TABLE 4.5

Comparison of reactor performance for plant cells

Reactor type	Oxygen transfer	Hydro-dynamic stress	Mixing	Scale-up	Limitations
Stirred-tank (ST)	High	Highly destructive	Completely uniform	Difficult	Cell death; contamination due to moving parts
ST-low agitation and modified impeller	Medium	Low	Reasonably uniform	Difficult	Insufficient mixing at very high cell densities
Bubble-column	Medium	Low	Non-uniform	Easy	Dead zones; settling of cells due to poor mixing
Air-lift	High	Low	Uniform	Easy	Dead zones at high cell densities
Rotating-drum	High	Low	Uniform	Difficult	Non-uniform mixing at very large scale

After Panda et al. (1989).

mid-1970s. Some of the bioreactors used for plant cell culture and their merits and demerits are listed in Tables 4.4 and 4.5.

Mostly the large scale plant cell cultures have been run as batch systems (Scragg, 1991). Up to 10-l bioreactors may be sterilized by autoclaving. In the case of larger reactors, the vessels are steam sterilized (2–3 exposures of 2 h each). Autoclaved or filter-sterilized medium is added to the vessel. Cultures are initiated with an inoculum ratio of about 1:10. Generally, the pH of the plant cell culture is not controlled. It is initially adjusted between 5 and 6. It often drops soon after inoculation to 4.5, rising slowly to 5–6 or above as growth proceeds. Temperature of the culture is generally maintained between 25° and 35°C. Growth optimum for *Catharanthus roseus* is 35°C (Scragg, 1991).

The major types of bioreactors currently in use for suspension culture of plant cells are stirred-tank, bubble column, air-lift, and rotating-drum reactors (Fig. 4.7).

(i) *Stirred-tank reactor*. The stirred-tank reactor (Fig. 4.7A), in which air is dispersed by mechanical agitation, represents the classical bioreac-
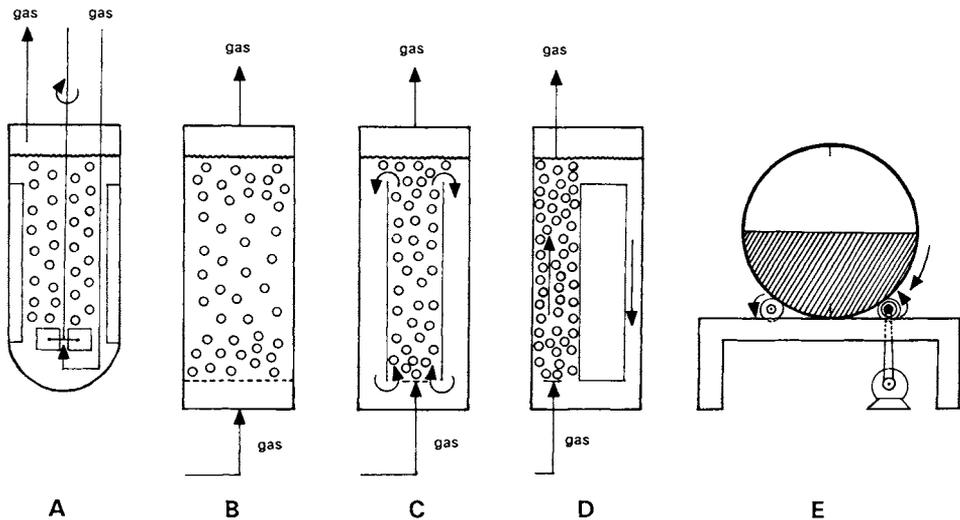
Fig. 4.7. Configuration of reactors used for plant cell cultivation. (A) Stirred-tank reactor; (B) bubble-column reactor; (C) air-lift reactor with draft-tube; (D) air-lift reactor with outer loop; (E) rotating-drum reactor.

tor for aerobic fermentations. Its behaviour has been well studied in a number of biological systems. Temperature, pH, amount of dissolved oxygen, and nutrient concentration can be controlled better within this reactor than any other reactor.

A major drawback of the stirred-tank reactor is the shearing stress generated by its stirring device to which plant cells may be sensitive. Despite this, most existing laboratory and commercial bioreactors are of the stirred-tank design which have been suitably modified for plant cell culture, such as: (1) the impeller speed is reduced to 50–150 rev. min^{-1} and in some cases the turbine impeller is replaced by a marine screen or paddle; (2) removal of baffles, pH probe and other probes not required; and (3) the sample ports are enlarged to about 1 cm to reduce blockage caused by cell aggregates. The two largest reactors (75 000 and 5000 l) used to date for plant cell culture are stirred-tank type (Scragg, 1994). For the first industrial plant cell culture process, in which shikonin was produced from *Lithospermum erythrorhizon*, stirred-tank reactors of 200 l and 500 l capacities were used (see Fig. 17.2; Tabata and Fujita, 1985). Cells of *Catharanthus roseus* have been grown in stirred-tank reactors by Wagner and Vogelmann (1977), Kurz et al. (1981), Pareilleux and Vinas (1983), Ducos and Pareilleux (1986) and Drapeau et al. (1986). Except for Wagner and Vogelmann, all others reported favourable results in terms of growth and product formation. It is interesting to note that some recent studies have revealed that many plant cell cultures are remarkably

tolerant to shear levels (1000 rps, shear rate 167 s^{-1}) once thought to be lethal (Scragg, 1994).

Some other disadvantages of stirred-tank reactors are their high energy requirements and complexity of construction and the fact that they are difficult to scale up.

(ii) *Bubble-column reactor.* The bubble-column reactor (Fig. 4.7B) is one of the simplest types of gas-liquid bioreactors used for aerobic fermentation. It consists of a cylindrical vessel aerated at the bottom. In such a system the gas is dispersed pneumatically through a deep pool of liquid by means of nozzles or perforated plates. A 1.5 kl bubble-column reactor has been used for the cultivation of *N. tabacum*, but insufficient mixing at such a scale reduced the specific growth rate of the cells.

Some of the merits of bubble-column reactor are: (i) it facilitates sterile operation because of the absence of moving parts and the fact that non-sealing parts are required, (ii) it provides high mass and heat transfer areas without the input of mechanical energy and may, thus, be suitable for shear-sensitive systems such as plant and animal cell culture, and (iii) the scale-up is relatively easy, and the reactor requires minimum maintenance.

The disadvantages of the bubble-column reactor are the undefined fluid flow pattern inside the reactor and its non-uniform mixing. Data on gas holdup and mass transfer characteristics for non-Newtonian fermentation are scanty.

(iii) *Air-lift reactor.* In air-lift reactor, as its name implies, compressed air is used for aeration and mixing of the contents of the reactor vessel. Its operation is based on the draught tube principle. Air sparged into the base of the reactor lowers the density of the medium which rises up the draft tube pulling fresh medium in at the base and, therefore, a flow is achieved. Schematic diagrams of the draught tube (inner loop and outer loop) air-lift vessels are given in Fig. 4.7C,D. A more uniform flow pattern is achieved in the air-lift reactor compared with the bubble column reactor, where a random flow pattern exists.

Air-lift reactors up to 100 l capacity have been used extensively by Fowler and his co-workers for cultivation of *C. roseus* cells. The cells of *Berberis wilsoneae, Cinchona ledgeriana, Cudrania tricuspidata, Dioscorea deltoidea, Digitalis lanata, Morinda citrifolia, Tripterygium wildordii* and, recently, *L. erythrorhizon* have also been cultured in air-lift reactors. In all cases, the reactor configuration gave favourable results in terms of biomass and product formation. Vienne and Marison (1986) have

reported successful continuous culture of *C. roseus* cells in an air-lift reactor.

The air-lift reactor is one of the most suitable bioreactor types for cultivation of plant cells on a large scale. It provides reasonable mixing and oxygen transfer at low shear, and less contamination occurs because there are no moving parts and no intrusion of impeller shaft. The operating cost, compared to the stirred-tank reactor, is low because of its simple design and it does not require power input for the stirrer. Despite these advantages air-lift reactors have not been used as extensively as stirred-tank reactors.

The disadvantages of air-lift reactors are the development of dead zones inside the reactor and insufficient mixing at high cell densities. Moreover, little information is available on the engineering analysis of the reactor behaviour in complex systems such as the plant cell cultures.

(iv) *Rotating-drum reactor.* The rotating-drum reactor consists of a horizontally rotating-drum on rollers connected to a motor (Fig. 4.7E). The rotating motion of the drum facilitates good mixing and aeration without imposing a high shear stress on the cultured cells. Baffles in the inner wall of the drum help to increase oxygen supply. This type of reactor has the capacity to promote high oxygen transfer to cells at high density. It has been used to grow cultures of *C. roseus* and *L. erythrorhizon* up to 1000 l in volume.

In a comparative study of the performance of rotating-drum and stirred-tank reactors for the cultivation of *Vinca rosea* the former was found to be superior on the basis of increased oxygen transfer at high cell densities (Tanaka et al., 1983). The rotating-drum reactor facilitates better growth and imparts less hydrodynamic stress. In the stirred-tank reactor growth rate was low at low agitation speed because of insufficient oxygen supply, while at high agitation speed the cells died. Hence, for cultivation of cells at high densities, the rotating-drum reactor was preferred. The rotating-drum reactor has also been shown to be superior to air-lift and modified stirred-tank reactors for the cultivation of *L. erythrorhizon* (Tanaka, 1987).

The major disadvantage of this reactor type is the restriction in scale-up.

(v) *Immobilized plant cell reactors.* Immobilization of plant cells into a suitable carrier and cultivation of the immobilized cells in different types of reactors has been developed as an alternative to free cell culture systems for the production of industrial phytochemicals (see Chapter 17). Entrapment in natural (alginate, agar, agarose, carrageenan) or syn-
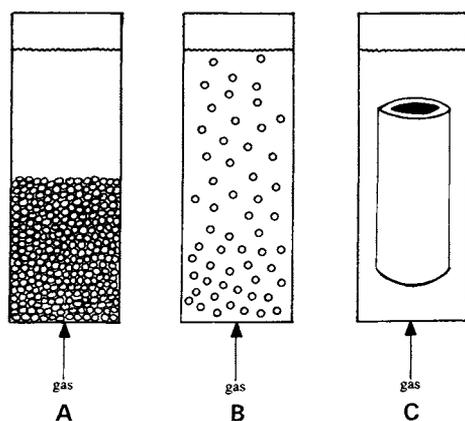
Fig. 4.8. Configuration of some reactors used to culture immobilized plant cells. (A) Packed-bed reactor; (B) fluidized-bed reactor; (C) polyurethane draft tube reactor.

thetic (polyacrylamide) polymers, adhesion to reticulate polyurethane foam, and confinement behind semi-permeable membranes have been employed to immobilize plant cells.

Alginate has been the most popular polymer used to immobilize plant cells. Cell suspension in 4% sodium alginate solution is allowed to fall as 2 mm drops in a beaker containing 0.2 M solution of $CaCl_2$. Ca-alginate is formed by ion exchange reaction and the drops harden as beads within 20–30 min. Alginate entrapped cells can be cultured in packed-bed (Fig. 4.8A), fluidized-bed (Fig. 4.8B) or air-lift bioreactors (Fig. 4.7C).

Polyurethane foam has been used to immobilize a range of cell lines. The cells are immobilized in these matrices by flowing cells and medium through the foam or by adding sterile foam to growing cultures. The foam can be cut into various shapes. Polyurethane entrapped cells have been cultured in both packed and fluidized beds as cubes, shaped into draft tube (Fig. 4.8C) or threaded as strips on stainless steel rods.

Membrane reactors (e.g. hollow-fibre units and flat membrane reactors), in which cells are separated from the growth medium by membrane, are particularly suitable for fragile cells which can be entrapped more readily on membrane and allow better control over cell density. The environment in a membrane reactor is more homogeneous; pressure drop and fluid dynamics are more easily controlled and are relatively independent of the scale of operation. In a hollow-fibre reactor (Fig. 4.9) cells are introduced into the shell side of the hollow-fibre cartridge and the medium is circulated through the fibre lumen and aerated using a separate reservoir. Since the cells do not stick to the fibre membrane the reactors may retain their mechanical integrity for a longer period of time and
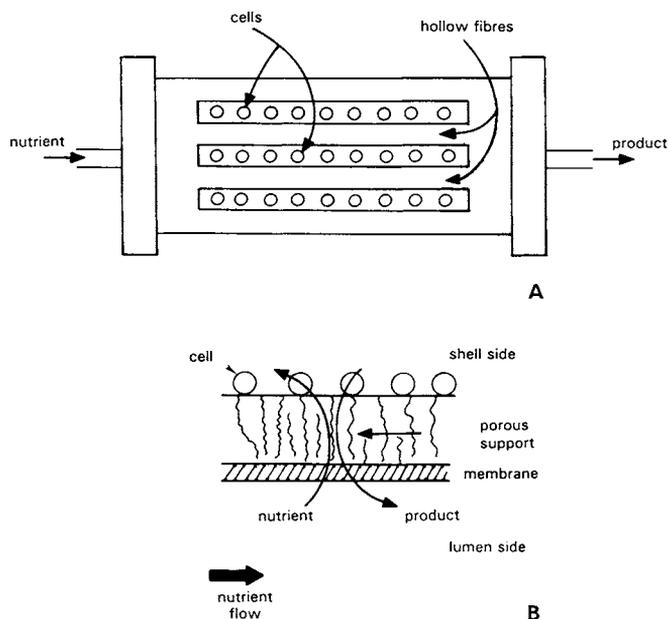
Fig. 4.9. (A) Hollow-fibre reactor for immobilized cell culture; (B) the portion marked in (A) enlarged to show the details of the reactor and the flow of nutrients and products across the membrane and the porous support of the hollow-fibre cartridge (adapted from Prenosil and Pederson, 1983).

may be reusable. When cells are no longer productive, when an experiment is over, or when a new cell-product combination is desired, it is potentially possible to flush out the old cells and refill the device with the new cells.

In flat-plate membrane reactor systems (Fig. 4.10), with one side flow and two side flow, the cells are loaded manually into the membrane cell layer and direct sampling can be achieved through a removal cap plate. Substrate enters the cell layer by diffusion or pressure driven flow and is converted into product which diffuses into the cell-free compartment. Multimembrane reactors have also been proposed for immobilized plant cell cultures. The main advantage of this reactor is that the desired metabolites are produced and selectively separated from the reactant simultaneously.

4.6. APPLICATIONS OF CELL CULTURE

4.6.1. Mutant selection

The occurrence of a high degree of spontaneous variability in cell cul-

tures and its exploitation in mutant selection in relation to crop improvement is discussed in Chapter 9. The frequency of certain phenotypes could be increased several fold by treating the cells with mutagens (Sung, 1976; Muller and Grafe, 1978; Miller and Hughes, 1980).

One of the major drawbacks of mutation breeding in higher plants is the formation of chimeras following the mutagenic treatment of multicellular organisms. In this regard cell culture methods of mutant selection are more efficient. Millions of potential plants can be handled in a minimal space; 100 ml of rapidly growing suspension cultures of tobacco contain over 1×10^7 cells.
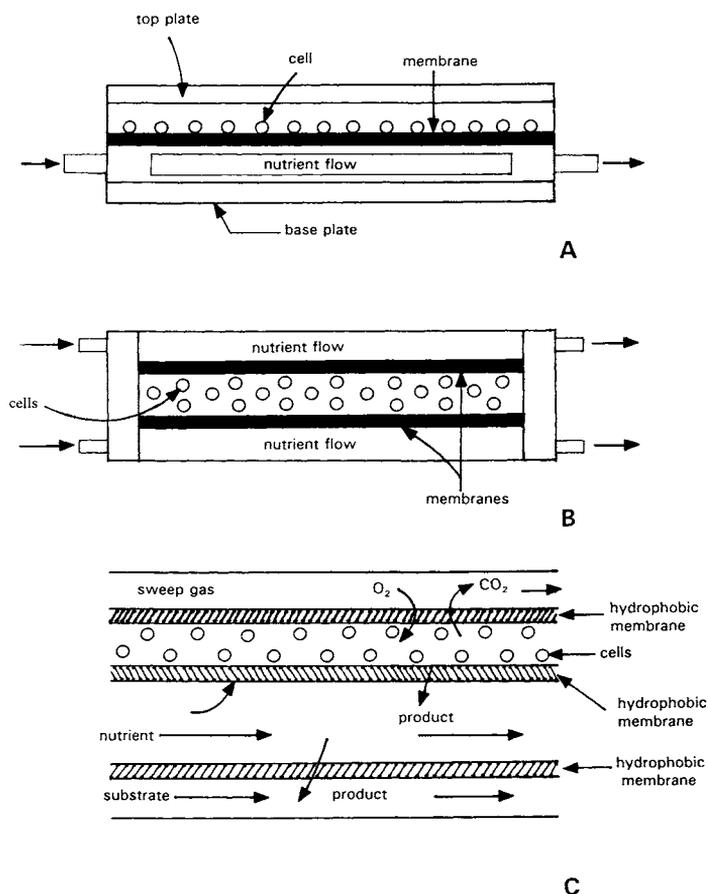

Fig. 4.10. Membrane reactor systems for immobilized plant cell culture. (A) Flat plate membrane reactor with one side flow of nutrients; (B) flat plate membrane reactor with two side flow of nutrients; (C) multimembrane reactor system. (A,B) are adapted from Shuler et al. (1984) and (C) from Shuler et al. (1986).

4.6.2. Industrial uses

Since the early 1950s many researchers have investigated the production of useful compounds by plant tissue cultures, and remarkable technological advances have been made to culture plant cells in large bioreactors for the commercial production of plant metabolites (see Chapter 17)

4.6.3. Induction of polyploidy

Doubling of chromosome number is frequently required to overcome the problem of sterility associated with hybrids of unrelated plants. In the genus *Saccharum* a large number of genetically sterile hybrids that exist could well be utilized in the breeding programmes if their fertility can be restored through doubling of their chromosome number (Heinz and Mee, 1970). Attempts using seeds and vegetative cuttings failed to accomplish this objective. Heinz and Mee (1970) demonstrated that a large number of polyploid plants of sugarcane could be produced through the use of cell cultures. They regenerated over 1000 plants from cell suspension cultures of a complex *Saccharum* species hybrid treated with 50 mg l^{-1} colchicine for 4 days. Cytological investigations revealed that about 48% of these plants were with uniformly doubled chromosome number. In this regard cell cultures should prove useful with other crop plants also.

Duplication of chromosomes in cell cultures also occurs spontaneously (Murashige and Nakano, 1966). This is one of the methods recommended for raising homozygous diploids from pollen-derived haploids (see Chapter 7).

4.7. CONCLUDING REMARKS

The methods of cell and callus culture are reasonably well developed. It is now possible to establish such cultures from most plant tissues. Several methods to culture plant cells in large bioreactors and at low plating densities or in complete isolation, under defined conditions, have been described (see also Chapter 12).

Potential applications of cell culture in mutant selection and the production of natural plant products are discussed in Chapters 9 and 17, respectively.

APPENDIX 4.I

Protocol for mechanical isolation of mesophyll cells from the leaves of
Calystegia sepium (after Rossini, 1972):

(a) Surface sterilize the leaves by rapid immersion in 95% ethanol
followed by rinsing for 15 min in filter-sterilized 7% solution of
calcium hypochlorite. Wash in sterile distilled water.

(b) Cut the leaves into small pieces (less than 1 cm^2).

(c) Homogenize 1.5 g of leaf material with 10 ml of culture medium
(for composition see Table 4.2) in a Potter-Elvehjem glass ho-
mogenizer tube.

(d) Filter the homogenate through two sterile metal filters, the upper
and lower filters with mesh diameters of 61 and 38 μm, respec-
tively.

(e) Fine debris can be removed by slow-speed centrifugation of the fil-
trate which would sediment the free cells. Remove the super-
natant and suspend the cells in a volume of the medium sufficient
to achieve the required cell density.

(f) Plate the cells in a thin layer of agar medium or liquid medium.

APPENDIX 4.II

Protocol for enzymatic isolation of mesophyll cells from tobacco leaves (after Takebe et al., 1968, as modified by Evans and Cocking, 1975):

(a) Take fully expanded leaves from 60–80-day-old plants and surface sterilize them by immersion in 70% ethanol for 30 s followed by rinsing for 30 min in 3% sodium hypochlorite solution containing 0.05% Teepol or cetavlon.

(b) Wash the leaves with sterile distilled water and peel off the lower epidermis with the aid of sterile fine jeweller's forceps.

(c) Excise peeled areas as 4 cm^2 pieces with a sterile scalpel blade.

(d) Transfer 2g of peeled leaf pieces to 100 ml Erlenmeyer flasks containing 20 ml filter sterilized enzyme solution containing 0.5% macerozyme, 0.8% mannitol, and 1% potassium dextran sulphate (MW source dextrin 560, sulphur content 17.3%; Meito Sangyo Co. Ltd., Japan).

(e) Infiltrate the enzyme into the leaf tissue by briefly evacuating the flasks with a vacuum pump.

(f) Incubate the flasks at 25°C for 2 h on a reciprocating shaker with a stroke of 4–5 cm at the rate of 120 cycles min^{-1}.

(g) Change enzyme solution after the first 30 min. The enzyme solution after the second 30 min should contain largely spongy parenchyma cells, and those after the third and the fourth 30 min periods should contain predominantly palisade cells.

(h) Wash the cells twice with culture medium and culture.