5

Cell Culture

Establishment of single cell cultures provides an excellent opportunity to investigate the properties and potentialities of plant cells. Such systems contribute to our understanding of the interrelationships and complementary influences of cells in multicellular organisms. The pioneering attempts made by Haberlandt (see Chapter 1) failed to achieve divisions in free cells but his detailed paper in 1902 stimulated further studies in this area. Subsequently, several workers reported spectacular success in achieving isolated single cell division and even raised complete plants from single cell cultures. This generated much interest among plant biotechnologists who recognised the merits of applying cell cultures over an intact organ or whole plant cultures to synthesise natural products. Using cell cultures in studies designed to describe the pathways of cellular metabolism was another aspect that initially attracted the attention of plant biologists. It was soon realised that single cell systems have a great potential for crop improvement. Free cells in cultures permit quick administration and withdrawal of diverse chemicals/substances, thereby making them easy targets for mutant selection. Moreover, the individual cells within a population of cultured cells invariably show cytogenetical and metabolic variations depending on the stage of the growth cycle and culture conditions. Such variability, termed 'spatial heterogeneity' (Lindsey and Yeoman 1985), has been the subject of much interest since differences between cells in their karyotype and the ability to accumulate secondary metabolites would manifest during morphogenesis in the clones regenerated from single cells. In this way the cell line selection technique can be usefully applied to produce high-yielding cultures as well as plants with superior agronomic traits.

5.1 Isolation of Single Cells

5.1.1 From Plant Organs

The most suitable material for the isolation of single cells is the leaf tissue since a more or less homogeneous population of cells in the leaves offer good candidates for raising defined and controlled large-scale cell cultures.

From such intact plant organs (as leaf tissue) single cells can be isolated using *mechanical* or *enzymatic* methods.

(I) MECHANICAL METHOD

Gnanam and Kulandaivelu (1969) developed a procedure which has since been successfully used to isolate mesophyll cells, active in photosynthesis and respiration, from mature leaves of several species of dicots and monocots including the grasses. Even metabolically active single cells from the bundle sheath of crab grass (*Digitaria sanguinalis*) can be isolated using a similar procedure. The procedure involves mild maceration of 10 g leaves in 40 ml of the grinding medium (20 μ mol sucrose, 10 μ mol $MgCl_2$, 20 μ mol tris-HCl buffer, pH 7.8) with a mortar and pestle. The homogenate is passed through two layers of muslin cloth and the cells thus released are washed by centrifugation at low speed using the same medium.

The mechanical isolation of free parenchymatous cells can also be achieved on a large scale. The details of this procedure are given in Appendix 5.1.

(II) ENZYMATIC METHOD

In 1968, Takebe and his co-workers treated tobacco leaf tissue with the enzyme pectinase and obtained a large number of metabolically active cells. A point to note is that potassium dextran sulphate in the enzyme mixture improved the yield of free cells (for details *see* Appendix 5.2).

Isolation of single cells by the enzymatic method has been found convenient as it is possible to obtain high yields from preparations of spongy parenchyma with minimum damage or injury to the cells. This can be accomplished by providing osmotic protection to the cells while the enzyme macerozyme degrades the middle lamella and cell wall of the parenchymatous tissue. Applying the enzymatic method to cereals (*Hordeum vulgare*, *Zea mays*) has proven rather difficult since the mesophyll cells of these plants are apparently elongated with a number of interlocking constrictions, thereby preventing their isolation.

5.1.2 From Cultured Tissues

The most widely applied approach is to obtain a single cell system from cultured tissues. Freshly cut pieces from surface-sterilised plant organs are simply placed on a nutrient medium (solidified) consisting of a suitable proportion of auxins and cytokinins to initiate cultures. Explants on such a medium exhibit callusing at the cut ends, which gradually extends to the entire surface of the tissue. The callus is separated from an explant and transferred to a fresh medium of the same composition to enable it

to build up a mass of tissue. Repeated subculture on an agar medium improves the friability of the callus, a prerequisite for raising a fine cell suspension in a liquid medium. The pieces of undifferentiated and friable callus are transferred in a continuously agitated liquid medium dispensed in autoclaved flasks or other suitable vials. Agitation is done by placing the culture flasks/vials on an *orbital-platform shaker* or suitable device. Movement of the culture medium exerts mild pressure on small pieces of tissue, breaking them into free cells and small cell aggregates. Further, it augments the gaseous exchange between the culture medium and the culture air, and also ensures uniform distribution of cells as well as cell clumps in the medium.

5.2 Growth and Subculture of Suspension Cultures

Cell suspensions are clonally maintained by the routine transfer (subculture) of cells in the early stationary phase to a fresh medium. During the incubation period the biomass of the suspension cultures increases due to cell division and cell enlargement. This continues for a limited period since the viability of cells in suspension after the stationary phase decreases due to the exhaustion of some factors or the accumulation of toxic substances in the medium. At this stage an aliquot of the cell suspension with uniformly dispersed free cells and cell aggregates is transferred to a fresh liquid medium of the original composition. The timing of subcultures is very important. The incubation period from culture initiation to the stationary phase is determined primarily by: (a) initial cell density, (b) duration of lag phase and (c) growth rate of cell line. The cell density used to subculture is critical and depends largely on the type of suspension culture to be maintained. Low initial cell densities will prolong the lag phase and exponential phases of growth. While initiating new suspension culture it is necessary to determine optimal cell density, proportionate to the volume of the culture medium, in order to achieve maximum growth. At an initial cell density of $9–15 \times 10^3$ ml^{-1}, the cells will generally undergo an eightfold increase in cell number before entering the stationary phase. Subcultures established with a high inoculum rate ($0.5–2.5 \times 10^5$ cells ml^{-1}) show an increase in cell number during the incubation period to a range $1–4 \times 10^6$ ml^{-1} before entering the stationary phase. The normal incubation time of stock cultures is 21–28 days between subcultures although cloning may occur within 18–25 days. In cases in which the cells are in a very active state of division, the passage length (periods between subcultures) may be reduced to 6–9 days. Cell cultures initiated at very low cell densities will not grow unless the medium is enriched with the metabolites necessary to grow single cells or a small population of cells.

5.3 Types of Suspension Cultures

5.3.1 *Batch Cultures*

These cultures are maintained continuously by propagating a small aliquot of the inoculum in the moving liquid medium and transferring it to a fresh medium (ca. 5X dilution) at regular intervals. Generally, cell suspensions are grown in flasks (100–250 ml) containing 20–75 ml of the culture medium. The biomass growth in batch cultures follows a fixed pattern (Fig. 5.1). When the cell number in suspension cultures is plotted against the time of incubation, a growth curve is obtained depicting that initially the culture passes through a lag phase, followed by a brief exponential growth phase—the most fertile period for active cell division. The growth declines after three to four cell generations, signalling that the culture has entered the stationary phase.

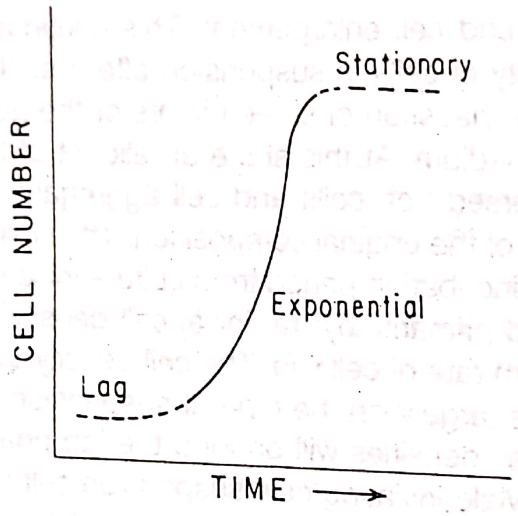

Fig. 5.1. Model curve showing different growth phases in batch cultures.

For a subculture, the flask containing the suspension culture is allowed to stand still for a few seconds to enable the large colonies to settle down. A pipette or a syringe with an orifice fine enough to hold aggregates of two to four cells or only single cells is used. The suspension is taken from the upper part of the culture and transferred to a fresh medium.

Batch cultures are characterised by a constant change in the pattern of cell growth and metabolism. As a result, batch cultures are not ideal systems for studies related to various aspects of cellular behaviour. In these cultures exponential growth with constancy of cell doubling time may be achieved, but there is no period of steady-state growth in which relative concentrations of metabolites and enzymes are constant. The drawbacks of batch cultures are overcome, to a certain extent, in *continuous cultures*.

5.3.2 Continuous Cultures

The large-scale cultures grown under steady state for long periods by adding fresh medium and draining out the used medium in a number of specially designed culture vessels are known as continuous or *mass* cultures. Continuous cultures are of the *closed* or *open* type. In the closed type, the addition of fresh medium is balanced by the outflow of old medium. The cells passing through the outflowing medium are separated mechanically and reintroduced in the culture. Cell biomass continues to increase as the growth proceeds. Paradoxically, the inflow of medium in the open type is accompanied by a balancing harvest of an equal volume of the culture medium and cells. This allows the indefinite maintenance of cultures at a constant and submaximal growth rate.

Basically there are two major types of open (continuous) cultures, viz., *chemostat* and *turbidostat*. The cell growth in chemostat cultures is maintained steady by a constant inflow of fresh medium consisting of nutrients (nitrogen, phosphorus, or glucose) at a concentration so as to be growth-limiting. Other constituents of such a medium are present at concentrations higher than required. Increase or decrease in the concentration of the growth-limiting factor is correspondingly expressed by increase or decrease in the growth rate of cells. Thus, the desired rate of cell growth can be maintained by adjusting the level of concentrations with respect to the growth-limiting factor and other constituents. In turbidostat cultures, on the contrary, the input of medium is intermittent as it is mainly required to control the rise in *turbidity* due to cell growth. The turbidity is preselected on the basis of biomass density in cultures and can be maintained by intermittent flow of medium and washout of cells.

A variety of rig configurations ranging from conventional stirred-tank reactors to bubble column and airlift systems (*see* Chapter 17) have been tested to achieve good mixing and homogeneous growth conditions that will not be deleterious to cell growth. Culture vessels are generally home made and manufactured principally of glass and/or stainless steel. A wide range of bioreactor configurations and sizes have been designed for continuous cultures depending on the variety of plant cells. These are described in detail by Martin (1980), Fowler (1982), and Panda et al. (1989).

Continuous cultures, besides commercial applications, offer certain other advantages: (a) ease of maintaining sterility over a long period of time, (b) less detrimental effects during mechanical failures, (c) a degree of automation and (d) versatility with regard to growth conditions such as temperature, aeration, stirring speed, illumination, nutrient and growth regulator levels. In spite of these advantages, plant tissue culturists refrain from using continuous cultures, probably because they require constant attention and specially designed equipment which pose practical rather than conceptual problems.

5.4 Culture Medium for Cell Suspensions

To obtain a fine suspension culture it is of prime importance that the callus used initially be friable. Moreover, the texture of a callus is genetically controlled and very often one experiences difficulty in achieving a good dispersion of cells. Manipulations in the media constituents and subculture routine may help in tissue dissociation although the addition to the medium of 2,4-D, small amounts of hydrolytic enzymes (cellulase and pectinase), or substances such as yeast extract, appears to have promotory effect on cell dispersion.

A good cell dissociation may also be achieved by permanently maintaining the cultures in the late lag phase by adding fresh medium every other day in a proportion that the biomass/medium volume is kept at 2. Sometimes it may be necessary to transfer small callus pieces or cell aggregates back to the agar or semi-solid medium. After two to three passages these pieces develop into a friable callus tissue which, on transfer to liquid medium, gives rise to a fine suspension.

Theoretically, the medium used for raising a fast-growing friable callus should prove suitable for initiating the cell suspension cultures of that particular species in a liquid medium. In practice, the requirements for rapidly growing cell suspensions differ from those for tissue or callus cultures. For example, the culture medium for a tobacco cell suspension requires an increase in the concentration of 2,4-D from 0.3 mg l^{-1} to 2 mg l^{-1}, followed by supplementing the callus medium with additional vitamins and casein hydrolysate (Table 5.1). Furthermore, the inorganic phosphate is rapidly utilised in actively growing suspension cultures and, consequently, becomes a limiting factor.

Table 5.1. Culture medium for tobacco suspension cultures[a]

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

[a] After Reynolds and Murashige (1979).

Many media have very little buffering capacity and the pH can change with an increase in the cell biomass. This necessitates monitoring and

adjustment of pH in suspension culture. B5 (*see* Chapter 3) and ER (Eriksson 1965) media are specially recommended for suspension culture of higher plants. These and other synthetic media are used for initial population density 5×10^4 cells ml^{-1} or more. With lower cell density, the medium requires to be *conditioned* or enriched with various other compounds.

5.4.1 *Conditioning of Medium*

In initiating cell cultures at low inoculum density a conditioned medium is used. A simple method is to filter out cells growing at high density from 4–6-week-old liquid cultures and to use this medium in drops or as thin layers to culture single cell/cells at low population density. The principle of conditioning followed by Torres (1989) involves the separation of a high-density cell culture from a low-density culture medium by a barrier that permits the diffusion of solutes and air. A high-density cell suspension (the nurse culture) kept inside a dialysis tube (Fig. 5.2) is suspended by means of a thread or rod in the flask containing the culture medium with low cell density (low-density medium). The metabolites produced by the nurse culture diffuse into the low-density medium, thereby increasing the latter's growth-promoting activity. This meets the conditions of growth for low cell populations since the necessary substances that may not be found in the low-density medium are released into it by the biosynthetic activity of the nurse cells.

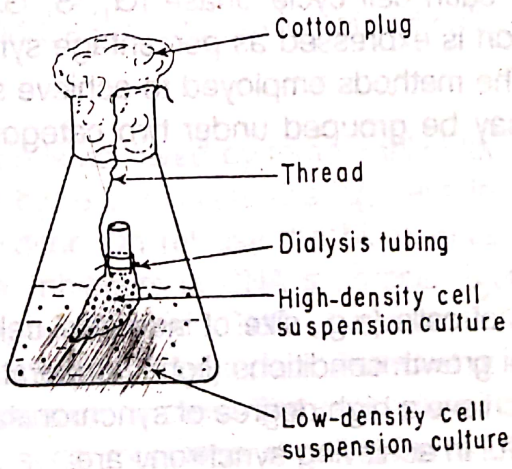

Fig. 5.2. Apparatus designed for conditioning of a low-density cell culture medium (modified from Torres 1989).

5.4.2 *Agitation of the Medium*

Suspension cultures require constant agitation of the medium for adequate aeration. This also facilitates dispersion of cells. It can be achieved using a shaker and suitable flasks. Muir (1953) was the first to introduce the orbital-platform shaker for growing suspension cultures of tobacco and

Tagetes erecta. The platform of the shaker is fitted with interchangeable clips of appropriate size for holding the flasks. A shaking speed of 30–150 rpm is optimum for most tissues. Rotary shakers are also used which have a disc that can be rotated at slow speed (1–2 rpm) by a shaft. About 10 ml medium is dispensed in a specially designed tube (12.5 cm long and 3.5 cm diameter) having a wide neck (1.7 cm diameter) and each tube mounted near the margin of the disc, the inoculum introduced from the neck and its mouth closed with a cotton plug. When the disc rotates, the cells and tissues are alternately bathed in the culture medium and thereby exposed to both nutrients and the culture air. Sometimes the tubes are substituted by special *nipple* flasks. Both orbital and rotary shakers have a control for regulating the speed.

5.5 Synchronisation of Suspension Cultures

Cells in suspension cultures vary greatly in size, shape, DNA and nuclear content. Moreover, the cell cycle time varies considerably within individual cells. Therefore cell cultures are mostly asynchronous. This variation complicates studies of biochemical, genetic, physiological and other aspects of cell metabolism. Hence it is essential to manipulate the growth conditions in asynchronous suspension culture in order to achieve a high degree of synchronisation. 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. Synchronisation is expressed as percentage synchrony of cells in suspension cultures. The methods employed to achieve synchronisation of suspension cultures may be grouped under two categories: *physical* and *chemical*.

5.5.1 Physical Methods

Physical properties of cells (e.g., size of individual cell/small cell aggregates) or environmental growth conditions (light, temperature) can be monitored successfully to achieve a high degree of synchronisation. Some of the physical methods helpful in achieving synchrony are:

(I) Selection by Volume

Synchronisation may be achieved on the basis of selecting the size of cell aggregates present even in the finest possible suspension cultures. This approach proved successful for carrot suspension cultures to the extent that 90% cell aggregates isolated were in early embryogenic stages. The procedure followed for selection of cell aggregates using the cell fractionation technique is given in Appendix 5.3.

(II) TEMPERATURE SHOCK

Low temperature shocks combined with nutrient starvation are reported to induce synchronisation of suspension cultures. This approach (*see* detailed procedure in Appendix 5.4) is now widely followed to increase the degree of cell synchronisation.

5.5.2 *Chemical Methods*

Cell cultures are *starved* of a nutrient or supplied with a biochemical *inhibitor* to prevent cells from completing a cell cycle. Through this approach the cells are first arrested at a particular stage of the cell cycle and, subsequently, allowed to undergo simultaneous divisions either by supplementing the starved chemical or withdrawing the inhibition.

(I) STARVATION

The principle of starvation is based on depriving suspension cultures of an essential growth compound leading to a stationary growth phase. Resupplying the missing compound is expected to induce resumption of cell growth synchronously. This procedure has been very effective for sycamore (*Acer pseudoplatanus*) suspension cultures. Cultures starved of nitrogen, phosphorus or carbonate, result in the arrest of cell growth during the G_1 or G_2 phase of the cell cycle. After a period of starvation, when these growth-limiting compounds are supplied to the medium, the stationary cells enter divisions synchronously (*see* Appendix 5.5). Growth hormone starvation is also reported to induce synchronisation of cell cultures.

(II) INHIBITION

Synchronisation is achieved by temporarily blocking the progression of events in the cell cycle and accumulating cells in a specific stage using a biochemical inhibitor. On release the block cells will synchronously enter the next stage. Inhibitors of DNA synthesis (5-aminouracil, FudR or 5-fluorodeoxypurine, hydroxyurea, TdR or excess thymidine) in cell cultures accumulate cells at the G_1/S boundary. Removal of the inhibitor is followed by synchronous division of cells. The procedure for using, a biochemical inhibitor to obtain synchronisation of cell cultures of *Haplopappus gracilis*, soybean, tobacco and tomato is described in Appendix 5.6.

(III) MITOTIC ARREST

Colchicine has been widely used to arrest cells at metaphase. Suspension cultures in exponential growth are supplied with 0.02% (w/v) colchicine for 4–8 hr in order to inhibit spindle formation. Longer colchicine treatment leads to an increased frequency of abnormal mitoses and chromosome stickiness. Colchicine should be filter-sterilised and only shorter duration treatment is recommended. This technique has been used for synchronisa-

tion of *Zea mays* suspension cultures but the likelihood of colchicine inducing genomic changes raises the possibility of obtaining asynchronous cultures.

5.6 Measurement of Growth in Suspension Cultures

Assessment of the growth in suspension cultures can be accomplished by following selected parameters at regular intervals. These include: (a) *cell counting*, (b) *packed cell volume* and (c) *fresh/dry weight increase of cells* and *cell colonies*.

5.6.1 Cell Counting

Cell count is a relatively more accurate measure adopted to determine the growth of cultures. Increase in cell number depends on the rate of mitotic index (MI) of cells in suspension cultures. Determination of cell number is a simple but tedious procedure since suspension cultures invariably carry cell colonies of various sizes. Therefore, it becomes essential to first disrupt cell aggregates by treating them with 5–15% chromic acid or pectinase (0.1% w/v, pH 3.5). The procedure described to count sycamore cells has been found suitable for cell counting. To 1 volume of cell suspension culture may be added 2 volumes of 8% chromic acid (trioxide) solution and the mixture heated at 70°C for 2–15 min. The mixture is cooled and then agitated vigorously for 10 min on a shaking machine. The suspension is now centrifuged, the chromic acid poured off and the pellet resuspended in 8% saline (NaCl) solution. After 10–15 min free cells are counted on a haemocytometer. Heating is avoided if an enzyme is used to disrupt cell aggregates.

5.6.2 Packed Cell Volume (PCV)

For PCV determination a small sample (10 ml) is removed from the uniformly disposed suspension culture aseptically and centrifuged in 15 ml-graduated conical centrifuge tubes at 1000 × g for 5 min. The packed cell volume is expressed as ml pellet ml^{-1} culture.

5.6.3 Cell Fresh Weight

The cells are collected on a preweighed (wet) circular filter of nylon fabric supported in a Hartley funnel, washed with distilled water under vacuum and the filter discs reweighed along with the cells.

5.6.4 Cell Dry Weight

A procedure similar to that for fresh weights is followed for determining cell dry weight except that the filter discs are dried in an oven for 12 hr at 60°C. After cooling in a desiccator containing silica gel, the dried filter is reweighed and the cell weight expressed as g ml^{-1} of culture or per 10^6 cells.

5.7 Viability of Cultured Cells

The growth of cultures is largely dependent on the viability of the cells, which can be assessed by microscopic examination of untreated cells or after staining them with substitute chemicals. These include:

5.7.1 Phase Contrast Microscopy

Cytoplasmic streaming and the presence of a healthy nucleus indicate that the cells are viable. Phase contrast microscopy is recommended as it is difficult to observe these aspects in unstained cells under a bright field.

5.7.2 Reduction of Tetrazolium Salts

This test is used to measure respiratory efficiency of cells by reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the red dye formazon. Formazon can be extracted and measured spectrophotometrically.

5.7.3 Fluorescein Diacetate (FDA) Method

The FDA method offers a quick visual assessment of the viability of cells. Stock solution of FDA (0.5% w/v) is prepared in acetone and stored at 0°C. Viability is tested by adding this solution to the cell or protoplast suspension at a final concentration of 0.01%. For protoplasts an appropriate osmotic stabiliser is added to the FDA solution. After 5 min incubation the cells are examined under a microscope with a suitable excitation or suppression filter. FDA, though non-fluorescing and non-polar, is cleaved by esterase activity inside the living cell, resulting in release of the polar portion of fluorescein, which fluoresces under UV. Since fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cells and thus becomes distinctly visible. In a dead cell the fluorescein is lost and remains invisible. Under UV light fluorescein gives a green fluorescence.

5.7.4 Evan's Blue Staining

This simple procedure is usually used as a complement to FDA. A dilute solution (0.025% w/v) of Evan's blue dye stains the dead or damaged cells while the living or viable cells repel the dye and remain unstained.

5.8 Culture of Isolated Single Cells

Free cells isolated either from plant organs (mesophyll tissue) or cell suspensions are grown as single cells under *in vitro* conditions using a suitable medium. This process, called *plating*, is of particular importance when attempting to obtain single-cell clones. Success in the culture of single cells, therefore, depends on the technique and various factors affecting cell plating.

5.8.1 *Plating Technique*

The technique developed by Bergmann (1960) is the most popular one for plating of single cells (Fig. 5.3). Free cells are suspended in liquid medium at a density twice the finally desired plating cell density. Equal volumes of the media containing single cells and a melted (30° to 35°C) agar medium (0.6–1%, w/v) are mixed and rapidly spread out in petri dishes in such a manner that the cells become fixed in an evenly distributed thin layer (ca 1 mm thick) after the agar has cooled and solidified. The dishes are sealed with parafilm and incubated in the dark or diffused light at 25°C. Free cells can also be plated in the liquid medium but follow-up of individual cells or their derivatives is difficult in this procedure because the cells do not remain in a fixed position. It is important to note that suspension cultures are filtered aseptically through a sieve that allows only the single cells required for culture to pass through; the cell aggregates are discarded.

5.8.2 *Plating Efficiency (PE)*

The plates or culture dishes may be observed under an inverted microscope and single cells marked on the outside of the plate with a fine marker to keep track of their regeneration potential. This ensures the isolation of pure single-cell clones. In preparing cultures, if a known volume of suspension is transferred to each plating dish, it should be possible to assess the PE quantitatively using the formula:

$$PE = \frac{\text{Final number of colonies/plate}}{\text{Initial number of cell units/plate}} \times 100$$

Usually, plating at cell densities of 10^3–10^5 cells ml^{-1} or more yields a high plating efficiency. Other parameters for obtaining a high PE are:

(a) Using a conditioned medium or synthetic medium designed to permit growth from a low initial density.
(b) Avoid plating cells held too long in stationary phase.
(c) Harvesting cells during exponential growth phase.
(d) Exposure of cells to temperature should never exceed 35°C.
(e) Incubating the plates in diffused light or darkness.

5.8.3 *Medium and Technique of Low-Density Cell Cultures*

(I) Medium

Efforts have been made to develop a synthetic medium for cells plated at low density. Cells plated in a culture medium synthesise necessary metabolites; when their concentrations reach a threshold value, a cell divides. This process of cells releasing metabolites into the medium continues until an equilibrium is reached between the cells and the medium. At initial high

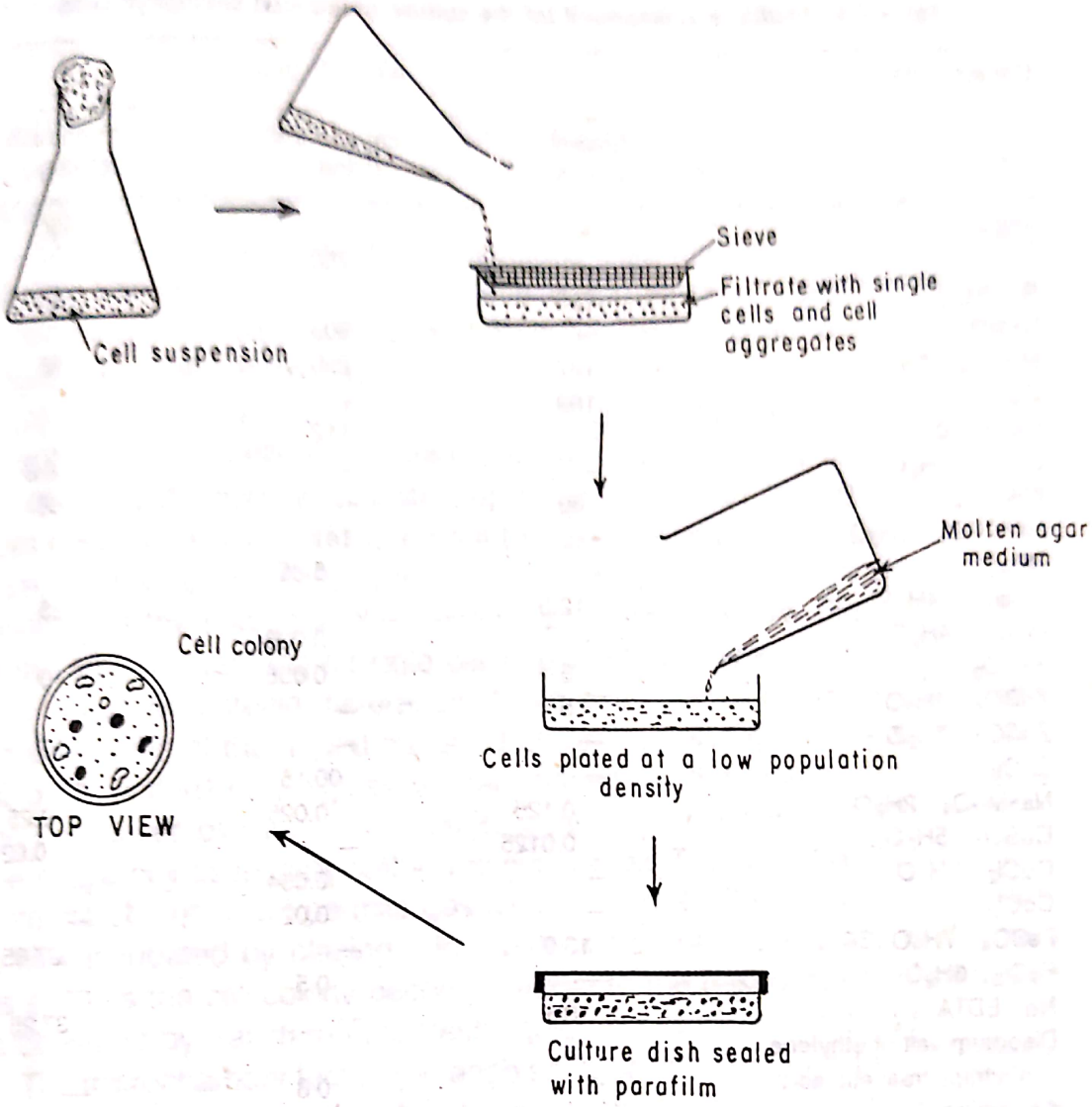

Fig. 5.3. Steps involved in Bergmann's technique of cell plating.

cell density the equilibrium is reached much earlier than at low cell density. Below a critical cell density the equilibrium is never reached and the cells fail to divide. This impediment to cell division, i.e., the population effect, can be overcome by supplementing the minimal medium with such undefined factors as coconut milk, casein hydrolysate and yeast extract. Various media developed for culturing isolated mesophyll cells are summarised in Table 5.2.

(II) TECHNIQUE

(a) *The filter-paper raft nurse technique*. The principle of this technique is similar to the conditioning of the culture medium described under Section 5.5.1. The basic difference is that a callus is used in place of the liquid medium to nurse the culture of an isolated single cell. Individual cells from a

Table 5.2. Media recommended for the culture of isolated mesophyll cells

Constituents	Amounts (mg l^{-1})		
	Rossini (1972)	Joshi and Ball (1968)	Kohlenbach (1984)
KNO_3	950	—	950
KCl	—	750	—
NH_4NO_3	725	—	720
$NaNO_3$	—	600	—
$MgSO_4 \cdot 7H_2O$	197	250	185
$CaCl_2$	169	—	—
$CaCl_2 \cdot 6H_2O$	—	112	—
$CaCl_2 \cdot 2H_2O$	—	—	16.6
KH_2PO_4	69	—	68
$NaH_2PO_4 \cdot 2H_2O$	—	141	—
NH_4Cl	—	5.35	—
$MnSO_4 \cdot 4H_2O$	12.5	—	25
$MnCl_2 \cdot 4H_2O$	—	0.036	—
H_3BO_3	5	0.056	10
$ZnSO_4 \cdot 4H_2O$	5	—	—
$ZnSO_4 \cdot 7H_2O$	—	—	10
$ZnCl_2$	—	00.15	—
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.025	0.25
$CuSO_4 \cdot 5H_2O$	0.0125	—	0.025
$CuCl_2 \cdot 2H_2O$	—	0.054	—
$CoCl_2$	—	0.02	—
$FeSO_4 \cdot 7H_2O$	13.9	—	27.85
$FeCl_3 \cdot 6H_2O$	—	0.5	—
Na · EDTA	18.6	—	37.25
Disodium salt of ethylene dinitrilotetraacetic acid	—	0.8	—
Gluvamine	—	—	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	—
Zn-Inositol	100	—	100
Adenine	—	—	20.25
BAP	0.1	—	—
Kinetin	—	0.1	1
2,4-D	1	1	1
Sucrose	10,000	20,000	10,000
pH	5.0	?	5.5

suspension culture or callus tissue (e.g., tobacco, marigold) are placed by means of a micropipette or spatula on top of an actively growing callus but separated by a filter-paper raft (see Fig. 1.1). After some days a cell, which normally fails to divide in the culture medium, is able to grow under the nurse effect of the callus. The whole operation is done aseptically and cell transfer should be rapid in order to avoid excessive drying of the cell and raft. Once a macroscopic colony develops from the cell on the filter-paper raft, it is transferred to an agar medium for further growth and maintenance under aseptic conditions. This method is now widely used to clone isolated single cells (see Chapter 1).

(b) *The microchamber technique*. De Ropp (1955) made the first attempt to culture single cells in a liquid medium using hanging drops. Success in obtaining divisions was limited up to the formation of aggregates of 10 cells or more, which could not meet the ultimate objective of raising clones from isolated single cells. Jones et al. (1960) accomplished this goal by developing the microchamber technique. In this method (Fig. 5.4), a drop of the medium carrying a single cell is placed on a sterile microscope slide and ringed with sterile mineral oil. Again one drop of mineral oil is placed on either side of the ringed culture drop and a cover glass placed on each oil drop. A third cover glass is then placed on the culture drop bridging the two cover glasses. As a result, a microchamber is formed enclosing the single cell aseptically within the mineral oil. The oil prevents water loss from the chamber but permits gaseous exchange. The microchamber slide is now incubated by placing it in a petri dish. The cover glass is removed as soon as the cell colony becomes visible to the naked eye and the tissue subcultured by transferring to fresh liquid or a semi-solid medium.

The microchamber technique enables visual monitoring of the divisions in an isolated cell. This method has been applied to raise a complete flowering plant of tobacco from a single cell in a culture medium containing mineral salts, sucrose, vitamins, Ca-pentothenate and coconut milk (see Chapter 1).

In conclusion, reasonable progress has been made to develop the methods of cell culture from most plant tissues. It is now possible to nurse isolated free cells *in vitro* at increasingly low plating densities under defined conditions. Due to the occurrence of a high degree of spontaneous variability in cultures, cloning of individual cells has the potential for application in mutant selection and synthesis of natural plant products. These aspects are detailed later in Parts III and IV.

in length have been found convenient for handling during transplantation. Longer roots may break in this process and lead to high mortality of transplanted plants.

16.7 Acclimatisation of Plants Transferred to Soil

Micropropagation on a large scale can be successful only when plants after transfer from culture to the soil show high survival rates and the cost involved in the process is low. Tissue culture plants generally show some structural and physiological abnormalities which inlcude: (a) abnormal leaf morphology and anatomy, (b) poor photosynthetic efficiency, (c) marked decrease in epicuticular wax and (d) malfunctioning of stomata. These characteristics as well as a heterotrophic mode of nutrition and a poor mechanism for water-loss control further render micropropagated plants vulnerable to transplantation shocks. Therefore, the transfer of individual plantlets to a potting mix and their acclimatisation under greenhouse conditions require the application of various methods to harden the plants/shoots for transplantation.

Plants are transferred to the soil usually after the *in vitro* rooting stage. However, the induction of *in vivo* rooting of cultured shoots may be more economical besides producing good quality roots. It is essential that the lower parts of tissue culture plants/shoots be washed thoroughly before their transfer to the potting mix (pumica, peat, vermiculite soil, sand, or their mixtures in different proportions). Transplanted plantlets or shoots are immediately irrigated with an inorganic nutrient solution and maintained under high humidity for the initial 10–15 days. This is required because plantlets during culture are adapted to almost 90–100% humidity.

High humidity can be built up around transplanted plants by covering them with clean transparent plastic bags having a small hole for air circulation. The size of the hole can be enlarged after two weeks in order to reduce the humidity. This enables shoots to adapt well to greenhouse conditions and to establish functional roots. Partial defoliation of plantlets and application of transpirants (1% Acropol, v/v) in the initial stages of transpiration reportedly improve the survival frequency due to reduction in water loss by plantlets. Most commercial laboratories these days have computerised hardening rooms with controlled conditions of light, temperature and humidity.

Direct transplantation of cultured plants (rice, tobacco) to the field has also been tried. Transplants survived with high frequencies provided a thin film comprising 50% aqueous glycerol and grease or paraffin (melting point 52-54°C) in an equal amount of diethylether, is applied on the surface of leaves with a brush before transplantation.

A major shock to plants, following transplantation, is the change from a substrate rich in organic nutrients to a substrate providing mostly inorganic nutrients. Attempts have been made to harden the shoot system by inducing autotrophism and development of surface wax on *in vitro* formed leaves. Increase in the epicuticular wax deposition could be induced either by exposing cultures (cabbage) to $CaCl_2$ or covering the medium with a thin layer of lanolin (chrysanthemum, cauliflower). These treatments also helped in reducing humidity. However, $CaCl_2$ or lanolin may prove detrimental to overall growth and development of plants. The relative humidity (RH) can also be reduced by opening the culture tubes inside a desiccator with $CaSO_4$ as the desiccant. This approach resulted in hardening of the tissue culture plants of carnation *in vitro* by development of wax after seven days. These plants then acclimatised easily and had a higher survival rate (96%).

Tissue culture plants of tree-legume species (*Leucaena leucocephala*) show high survival rates provided a pretransplant is introduced. In this stage, plants are transferred to screw-cap bottles containing sterilised quartz sand irrigated with an inorganic nutrient solution carrying an efficient strain of *Rhizobium* (NGR 8). Bottles are initially kept closed for two weeks and subsequently the caps removed to maintain plants under controlled conditions of light and temperature ($25 \pm 2°C$ and 18 Wm^{-2}) for another two weeks before their final transfer to the field.

Storage organs have been induced in cultured shoots of several species. These structures do not require hardening and can be directly transplanted to the soil with survival rates comparable to a similar type of organs formed *in vitro*. The advantage of *in vitro* tuberisation is that the additional step of rooting the shoots is altogether eliminated. A well-known example is the production of aerial tubers by cultured shoots of potato under the influence of chloramequat. Other examples in which *in vitro* tuberisation can be induced are *Dioscorea bulbifera, D. alata* and *D. rotundata*. Formation of bulblets in cultures of *Muscari armeniacum* and *Narcissus tazetta* was observed to occur in the presence of activated charcoal, while a high sucrose concentration appeared critical for *in vitro* cormlet formation in *Gladiolus* shoots. Transplantation of micropropagated plants of *Gladiolus* has been a serious problem. Corms developed *in vitro* can circumvent this problem as they show high germination (75–80%) under field conditions (for details and references see Bhojwani and Dhawan 1989).

16.8 Clonal Multiplication of Woody Species

given in Chapter 18.

7.5.2 Synthesis of Synthetic/Artificial Seeds

There is considerable worldwide interest in the development of methods for encapsulation of somatic embryos to enable them to be sown under field conditions as 'synthetic' or 'artificial' seeds. Research programmes on production of artificial seeds via somatic embryogenesis in respect of commercially important crops would not only contribute to increased agricultural production, but also add to our basic knowledge of the regulatory mechanisms which control plant growth and differentiation. The concept is now extended to encapsulation of protocorm-like bodies, shoot tip, shoot buds, etc. which can be used as clonal seeds (Khor and Loh 2005).

Synthetic seeds, consisting of somatic embryos enclosed in a protective coating, have been proposed as a 'low-cost-high-volume' propagation system. The inherent advantages of synthetic seeds are the production of many somatic embryos and the use of conventional seed-handling techniques for embryo delivery. The objective is to produce clonal 'seeds' at a cost comparable to true seeds. Two types of synthetic seeds have been developed, namely, *hydrated* and *desiccated*. Redenbergh et al. (1986) developed hydrated artificial seeds by mixing somatic embryos of alfalfa, celery and cauliflower with sodium alginate, followed by dropping into a solution of calcium chloride/nitrate to form calcium-alginate beads. About 29-55% embryos encapsulated with this hydrogel germinated and formed seedlings *in vitro*. Kim and Janick (1989) applied synthetic seed coats to clumps of carrot somatic embryos to develop desiccated artificial seeds. They mixed equal volumes

of embryo suspension and 5% solution of polyethylene oxide (polyox WSR N-750), a water-soluble resin, which subsequently dried to form polyembryonic desiccated wafers. The survival of encapsulated embryos was further achieved by embryo 'hardening' treatments with 12% sucrose or 10^{-6}M ABA, followed by chilling at high inoculum density.

Calcium alginate capsules tend to stick together and are difficult to handle because they lose water rapidly and dry down to a hard pellet within a few hours of exposure to the atmosphere. These problems can be offset by coating capsules with Elvax 4260 (ethylene vinyl acetate acrylic acid tetrapolymer; Du Pont, USA). From a practical sowing situation, it is necessary to produce high and uniform quality synthetic seeds at large scale. For this an automate encapsulation process has been developed. Its protocol has been described by Onishi et al. (1994) and Sakamoto et al. (1995). Processes for use of microcapsules (that release sucrose inside alginate beads), or self-breaking beads, pharmaceutical type capsules, and cellulose acetate mini-plugs, have been recently developed to promote sowing of coated somatic embryos as artificial seeds as well as their germination in non-sterile environment, such as in greenhouse or directly in the field. These techniques of embryo coating are summarised in an overview of synthetic seed germination by Dupuis et al. (1999).

Another delivery system for somatic embryos for obtaining transgenic plants is *fluid-drilling*. Embryos are suspended in a viscous-carrier gel which extrudes into the soil. The primary problem in fluid-drilling is that the sucrose level necessary to permit conversion also promotes rapid growth of contaminating micro-organisms in a non-aseptic system. Gray (1987) found that somatic embryos of orchard grass (*Dactylis glomerata*) became quiescent when desiccated in empty plastic petri dishes at 70% relative humidity at 23°C which amounted to loss of 13% water. However, after 21 days of storage, desiccated embryos when rehydrated *in vitro* germinated to produce viable plantlets though limited (4%) in number. Senaratna et al. (1990) treated alfalfa somatic embryos with ABA at the torpedo to cotyledonary stages in order to increase their tolerance to desiccation. Over 60% of such desiccated embryos germinated when placed on a moist filter paper or sown directly onto sterile soil and formed plantlets. Heat-shock treatments, osmotic stress and nutrient deprivation also induce a degree of desiccation tolerance comparable to that conferred by ABA treatment and have no detrimental effect on the subsequent growth of the plantlets (Thorpe and Stasolla 2001). For efficient methods of synthetic seed production and its conversion to plantlets for clonal propagation in *Catharanthus*, *Dendrobium* and *Stevia* refer to Maqsood et al. (2012), Siew et al. (2014) and Nower (2014), respectively.

7.5.3 Source of Regenerable Protoplast System

Embryogenic callus, suspension cultures and somatic embryos have been employed as sources of protoplast isolation for a range of species. Cells or tissues in these systems have demonstrated the potentiality to regenerate in cultures and, therefore, yield protoplasts that are capable of forming whole plants. Embryogenic cultures are especially valuable in providing a source of regenerable protoplasts in the graminaceous coniferous and citrus species. Attempts t achieve regeneration of callus or even sustaine divisions in mesophyll-derived protoplasts Gramineae proved unsuccessful until Vasil an Vasil (1986) turned to embryogenic cultur obtained from immature embryos of pe millet (*Pennisetum purpureum*) as the source protoplasts. Protoplasts from these cultu were induced to divide to form a cell m from which embryoids, and even plantl regenerated on a suitable nutrient medi Similar success was subsequently reporte other workers with embryogenic suspensio *Panicum maximum*, *Pennisetum purpureum*, C *sativa*, *Saccharum officinarum*, *Lolium per Festuca arundinaceae* and *Dactylis glom*

subcultured by transferring to fresh liquid or a semi-solid medium.

The microchamber technique enables visual monitoring of the divisions in an isolated cell. This method has been applied to raise a complete flowering plant of tobacco from a single cell in a culture medium containing mineral salts, sucrose, vitamins, Ca-pentothenate and coconut milk (*see* Chapter 1).

5.8.4 Bioreactor for Large Scale Culture

Mostly large scale cultivation of plant cells has been achieved as continuous cultures, which requires bioreactor configurations of various sizes (*see* Section 5.3.2). A bioreactor is a glass or steel vessel fitted with probes to monitor the pH, temperature and dissolved oxygen in the cell culture under aseptic conditions. Bioreactors have found increasing applications in industrial production of valuable compounds (Scragg 1994, 1999, Eibl and Eibl 2008, Ruffoni et al. 2010). The details of design regarding bioreactors used in cell cultures are discussed in Chapter 17.

In conclusion, reasonable progress has been made to develop the methods of cell culture from most plant tissues. It is now possible to nurse isolated free cells *in vitro* at increasingly low plating densities under defined conditions. Due to the occurrence of a high degree of spontaneous variability in cultures, cloning of individual cells has the potential for application in mutant selection and synthesis of natural plant products. These aspects are detailed later in Parts III and IV.

The plant cell cultures currently have increasing role in large-scale production of pharmaceutical proteins. Protalix Biotherapeutics develops recombinant proteins and produces them in plant cell culture. Teliglucerase alfa is reported as the first biotherapeutic protein expressed in plant cells and is now under approval for commercial use in the world. Other therapeutic proteins being developed for production in plant cell cultures and major milestones reached by protalix biotherapeutics are summarised in a review by Tekoah et al. (2015).