Mechanical separation. In some plant species, disrupting the tissue mechanically can separate intact cells of certain organs. Viable mesophyll cells, for example, can be obtained easily from *Asparagus cladodes* (Colman *et al.,* 1979) and from leaves of *Macleaya cordata* (Kohlenbach; 1966, 1967). These cells can be grown either in suspension or solid culture and induced into morphogenesis, including somatic embryo formation (Kohlenbach, 1977). Schwenk (1980, 1981) simply placed pieces of the young cotyledons of sweet potato in water inside an abrasive tube in which a vortex was created. After removing debris, a cell suspension could be obtained from which cells grew and formed callus when plated on nutrient agar.

However, the capacity to isolate separated cells directly from higher plants appears to be limited (Jullien and Rossini, 1977). The type of tissue used seems to be important both to permit cell separation and to obtain subsequent growth. Cells separated from the leaves, instead of from the cotyledons, of sweet potato (above) had no capacity for growth, and it was not possible to even separate cells by mechanical means from several other plants.

Enzymatic separation. Cell separation can be assisted by treating plant tissue with enzyme preparations such as crude pectinase or polygalacturonase, which loosen the attachment between individual cells in a tissue. Zaitlin first used this technique in 1959 to separate viable cells from tobacco leaves. Methods of isolation have been described by Takebe *et al.* (1968); Servaites and Ogren (1977) and Dow and Callow (1979). Cells isolated in this way can be suspended in culture medium and remain metabolically active.

Separated cells from leaf tissue of tobacco pre-infected with Tobacco Mosaic Virus have been used to study the formation of viral RNA's in the infected cells, and for studies on the interaction between leaf tissue cells and elicitor chemicals produced by fungal pathogens (Dow and Callow, 1979). Button and Botha (1975) produced a suspension of single cells of *Citrus* by macerating callus with 2-3% Macerase enzyme: the degree of dispersion of cells from suspension cultures can also be improved by enzyme addition (Street, 1977c).

3.3.3. Protoplasts

A protoplast is the living part of a plant cell, consisting of the cytoplasm and nucleus with the cell wall removed. Protoplasts can be isolated from whole plant organs or tissue cultures. If they are then placed in a suitable nutrient medium, they can be induced to re-form a cell wall and divide. A small cluster of cells eventually arises from each cell and, providing the protoplasts were originally plated at a relatively low density, can be recognised as one of many discrete 'callus colonies'. Plants can often be regenerated from such callus. Protoplast culture therefore provides one route whereby plants can be multiplied, but it is not yet used for routine micropropagation work, although the number of species in which plant regeneration has been achieved is steadily increasing.

At present isolated protoplasts are used chiefly in research into plant virus infections, and for modifying the genetic information of the cell by inserting selected DNA fragments. Protoplasts may also be fused together to create plant cell hybrids. Genetically modified cells will be only of general practical value if whole plants having the new genetic constitution can be regenerated from them. The ability to recover plants from protoplast cultures is therefore of vital importance to the success of such genetic engineering projects in plant science.

Methods of protoplast preparation. There are several different methods by which protoplasts may be isolated:

- by mechanically cutting or breaking open the cell wall;
- by digesting away the cell wall with enzymes;
- by a combination of mechanical and enzymatic separation.

For successful isolation it has been found essential to cause the protoplast to contract away from the cell wall, to which, when the cell is turgid, it is tightly adpressed. Contraction is achieved by plasmolysing cells with solutions of salts such as potassium chloride and magnesium sulphate, or with sugars or sugar alcohols (particularly mannitol) (see Chapter 4). These osmotica must be of sufficient concentration to cause shrinkage of the protoplasm, but of insufficient strength to cause cellular damage.

In the past, protoplasts have been mechanically isolated from pieces of sectioned plant material, but only very small numbers were obtained intact and undamaged. This method has therefore been almost completely replaced by enzymatic isolation techniques. Commercially available preparations used for protoplast isolation are often mixtures of enzymes from a fungal or bacterial source, and have pectinase, cellulase and/or hemicellulase activity: they derive part of their effectiveness from being of mixed composition (Evans and Cocking, 1977).

Protoplasts are usually isolated using a combination of several different commercial products. Plasmolysis helps to protect the protoplast when the cell wall is ruptured during mechanical separation and also appears to make the cell more resistant to the toxic effects of the enzymes used for cell wall digestion. It also severs the plasmodesmata linking adjacent cells and so prevents the amalgamation of protoplasms when the cell walls are digested away.

Tissue from an entire plant to be used for protoplast separation, is first surface sterilised. Some further preparation to allow the penetration of osmotic solutions and the cell wall degrading enzymes, is often advantageous. For instance, when protoplasts are to be separated from leaf mesophyll, the epidermis of the leaf is first peeled away, or the leaf is cut in strips and the tissue segments are then plasmolysed. The next step is to incubate the tissue with pectinase and cellulase enzymes for up to 18 hours in the same osmoticum, during which time the cell walls are degraded. Agitation of the incubated medium after this interval causes protoplasts to be released. They are washed and separated in solutions of suitable osmotic potential before being transferred to a culture medium.

Less severe and prolonged enzymatic cell digestion is required if plant tissue is first treated to mild mechanical homogenisation before cellulase treatment. Another technique calls for the sequential use of enzymes; firstly pectinase to separate the cells, and then, when separation is complete, cellulase to digest the cell walls. The yield of viable protoplasts can sometimes be increased by pre-treatment of the chosen tissue with growth substances before separation is attempted (Kirby and Cheng, 1979). Protoplasts are also commonly isolated by enzymatic treatment of organs or tissues that have been cultured *in vitro*. Cells from suspension cultures, which have been subcultured frequently, and are dividing rapidly, are one suitable source.

The successful isolation of viable protoplasts capable of cell division and growth, can depend on the manner in which the mother plant was grown. For example, Durand (1979) found that consistently successful protoplast isolation from haploid *Nicotiana sylvestris* plants depended on having reproducible batches of young plants *in vitro*. The composition of the medium on which these plants were cultured had a striking effect on protoplast yield and on their ability to divide. A low salt medium devoid of vitamins was particularly disadvantageous. The light intensity under which the plants were grown was also critical.

Protoplast culture. Isolated plant protoplasts are very fragile and particularly liable to either physical or chemical damage. Thus if they are suspended in a liquid medium, it must not be agitated, and the high osmotic potential of the medium in which isolation was carried out must be temporarily maintained. As growth depends on adequate aeration, protoplasts are usually cultured in very shallow containers of liquid or solid media; fairly high plating densities (5×10^4 to 10^5 protoplasts/ml) may be necessary, possibly because endogenous chemicals are liable to leak away from such unprotected cells. To promote growth, it may also be beneficial to add to the medium supplementary chemicals and growth factors not normally required for the culture of intact cells.

The capability of plant protoplasts to divide appears to be closely related to their ability to form a cell wall (Meyer and Abel, 1975a,b). The type of wall that is produced initially can be controlled to some extent by the nature of the culture medium. A non-rigid wall can be produced on tobacco mesophyll protoplasts, for example, by culture in a medium containing a relatively high concentration of salts; but although such cells will divide 2–3 times, further cell division does not occur unless a rigid wall is induced to be formed by a change in the culture medium (Meyer, 1974). Under favourable circumstances formation of a cell wall seems to occur as soon as protoplasts are removed from hydrolysing enzyme preparations, and the first signs of cellulose deposition can be detected after only about 16 hours in culture medium. Once wall formation is initiated, the concentration of osmoticum is reduced to favour cell growth. This is readily accomplished in a liquid medium, but where protoplasts have been plated onto a solidified medium it will be necessary to transfer the cells on blocks of agar, to another substrate.

When it has formed a cell wall, the regenerated plant cell generally increases in size and may divide in 3–5 days. If further cell divisions occur, each protoplast gives rise to a small group of intact cells and then a small callus colony. Green chloroplasts in cells derived from leaf mesophyll protoplasts, lose their integrity and disappear as callus formation proceeds. Protoplasts may originate from cells of the intact plant, which are not all of the same genetic composition. If such cells are grown in liquid medium, they may stick together and form common cell walls. Colonies of mixed callus will result which

could give rise to genetically different plants (see Chapter 3) or plant chimeras (D'Amato, 1978).

To avoid cell aggregation, protoplasts should be freely dispersed and cultured at as low a density as possible. This may mean that, as in the culture of intact cells at low density (see above), nurse tissue, or a conditioned or specially supplemented medium, must be employed. A method of the latter kind was devised by Raveh *et al.* (1973). A fabric support has been used to suspend protoplasts in a liquid medium so that media changes can be made readily (Kirby and Cheng, 1979).

For further information, readers should consult one or other of the following references:

- Bajaj (1977), Evans and Cocking (1977) and Evans and Bravo (1983), who provide good basic reviews of the subject
- Gamborg *et al.* (1981), describe methods and protocols for protoplast isolation, culture (and fusion)
- Constabel (1982) and Fowke (1982a), chapters describing methods and equipment for protoplast isolation and culture

An entire plant was first regenerated from callus originated from an isolated protoplast in 1971 (Takebe *et al.,* 1971). Since then plants have been produced from the protoplasts of a wide range of species, using indirect shoot morphogenesis or indirect embryogenesis (Davey and Power, 1988). The direct formation of somatic embryos (see below) from cultured protoplasts is also possible (Zapata and Sink, 1980).

Protoplast fusion. Although fusion of plant protoplasts was observed many years ago, it has become especially significant since methods have been developed for protoplast isolation and subsequent regeneration into intact plants. Isolated protoplasts do not normally fuse together because they carry a superficial negative charge causing them to repel one another. Various techniques have been discovered to induce fusion to take place. Two of the most successful techniques are the addition of polyethylene glycol (PEG) in the presence of a high concentration of calcium ions and a pH between 8-10, and the application of short pulses of direct electrical current (electro-fusion). By mixing protoplasts from plants of two different species or genera, fusions may be accomplished:

- (a) between protoplasts of the same plant where fusion of the nuclei of two cells would give rise to a homokaryon (synkaryon);
- (b) between protoplasts of the same plant species (intravarietal or intraspecific fusion);
- (c) between protoplasts of different plant species or genera (interspecific or intergeneric fusion).

Fusions of types (b) and (c) above can result in the formation of genetic hybrids (heterokaryocytes), which formally could only be obtained rarely through sexual crossings. By separating the fused hybrid cells from the mixed protoplast population before culture, or by devising a method whereby the cells arising from fused cells may be recognised once they have commenced growth, it has been possible to regenerate new somatic hybrid (as opposed to sexually hybrid) plants. Some novel interspecific and intergeneric hybrid plants have been obtained by this means. A fusion of the cytoplasm of one kind of plant with the nucleus of another is also possible. Such cybrid plants can be useful in plant breeding programmes for the transfer of cytoplasmic genes.

The following references give further details about this research topic and its implications for crop improvement:

- Schieder and Vasil (1980). A well-referenced review which lists somatic hybrid cell lines or plants obtained by protoplast fusion.
- Ferenczy and Farkas (1980) is a book on protoplast research in fungi, yeasts and plants. Several papers describe the results of fusions between protoplasts of different plant species or genera.
- Dodds and Roberts (1982), a short chapter describing methods and techniques.
- Keller *et al.* (1982), a useful review of the production and characterisation of somatic hybrids and the practical applications of protoplast fusion technology.
- Kao (1982) and Fowke (1982a,b) describe protocols for protoplast fusions in great detail.
- Mantell *et al.* (1985). An introduction to plant genetic engineering of various kinds.
- Glimelius (1988). Uses of protoplast fusion for plant breeding objectives.
- Davey and Power (1988). Progress in protoplast culture, fusion and plant regeneration

4. CYTODIFFERENTIATION

In an intact plant there are many kinds of cells all having different forms and functions. Meristematic cells, and soft thin-walled parenchymatous tissue, are said to be undifferentiated, while specialised cells are