Chapter 12

Protoplast Isolation and Culture

12.1. INTRODUCTION

In eukaryotes the transfer of genetic material from one individual to another is conventionally achieved through sexual breeding, the scope of which is extremely limited, particularly in animals. Even in plants, where fairly distant species could be crossed, it has not always been possible to obtain full hybrids between desired individuals because of sexual incompatibility barriers (see also Chapters 10 and 11). This has often proved a serious handicap in crop improvement programmes through hybridization. In this respect cell fusion offers a novel approach to distant hybridization (somatic hybridization). Fusion of cells, whether in plants or animals, must occur through the plasma membrane. Unlike animals, in plants the plasma membrane is bound by a rigid cellulosic wall, and the adjacent cells are cemented together by a pectin-rich matrix. It is mainly for this reason that somatic cell genetics is more advanced with animal than with plant systems. It is only since 1960, when E.C. Cocking at the University of Nottingham demonstrated the feasibility of enzymatic degradation of plant cell walls to obtain large quantities of viable naked cells, hereafter called protoplasts[1], that real interest in genetic modification of somatic cells in higher plants has developed. Actually, active contributions in this area started appearing after 1970.

Besides being able to fuse with each other, higher plant protoplasts can also take up foreign DNA, through their naked plasma membrane, under specific chemical and physical treatments. However, the importance of an isolated protoplast system in genetic transformation of plants has been somewhat eclipsed by more recent techniques of gene insertion into intact plant cells such as co-cultivation of explants with disarmed vectors of *Agrobacterium* and particle gun (see Chapter 14).

Protoplasts also provide an experimental system for a wide range of biochemical and molecular studies ranging from investigations into the growth properties of individual cells to membrane transport.

[1] Torrey and Landgren (1977) have defined higher plant protoplasts as 'cells with their walls stripped off and removed from the proximity of their neighbouring cells.'

Essential ingredients of the technique of genetic modification of plant cells through the protoplast system are: (a) isolation of protoplasts, (b) culture of protoplasts to raise whole plants, (c) cell fusion, and (d) introduction of foreign genetic material into the protoplasts. This chapter deals with the techniques of isolation and culture of protoplasts. Cell fusion in relation to somatic hybridization and genetic transformation of plants using isolated protoplasts are discussed in Chapters 13 and 14, respectively.

12.2. ISOLATION OF PROTOPLASTS

The isolation of protoplasts from higher plants was pioneered by Klercker in 1892. The procedure followed by him was largely mechanical; the cells were kept in a suitable plasmolyticum and cut with a fine knife. In this process some of the plasmolyzed cells were cut only through the cell wall, releasing intact protoplasts. This procedure for protoplast isolation is applicable only to vacuolated cells. Another limitation of this mechanical method is that the yields are extremely low.

In 1960, Cocking demonstrated the possibility of enzymatic isolation of a large number of protoplasts from cells of higher plants. He used a concentrated solution of cellulase enzyme, prepared from cultures of the fungus *Myrothecium verrucaria*, to degrade the cell walls. However, real progress in this area was made after 1968 when cellulase and macerozyme enzymes became available commercially.

The commercial preparations of the enzymes for protoplast isolation were first employed by Takebe et al. (1968). In the scheme followed by these workers to isolate mesophyll protoplasts of tobacco, the two enzymes were used sequentially. The leaf pieces were first exposed to macerozyme to liberate single cells which were then treated with cellulase to digest the cell walls and release the protoplasts. Power and Cocking (1968) demonstrated that the two enzymes can be used together. This 'simultaneous' or 'one step' method is faster than the sequential method and reduces the chances of microbial contamination by cutting down a few steps. Most workers now use this simplified one-step method (for example see Appendix 12.I). A range of enzyme preparations are now available commercially (Table 12.1), and, depending on the nature of the tissue these are used in different combinations.

The use of commercially available enzymes has enabled the isolation of protoplasts from virtually every plant tissue, as long as the cells have not acquired lignification. Protoplast isolation has been reported from mesophyll cells of in vivo and in vitro growing plantlets, aseptic seedlings, microspore mother cells, young microspores, pollen grain calli, and embryo-

TABLE 12.1

Some commonly used commercially available enzymes for protoplast isolation

Enzyme	Source	Supplier
Cellulases		
Onozuka RS	*Trichoderma viride*	Yakult Honsha, Japan
Cellulase R-10	*T. viride*	Yakult Honsha, Japan
Cellulysine	*T. viride*	Calbiochem, USA
Driselase	*Irpex lactes*	Kyowa Hakko Kogyo, Japan
Meicelase-P	*T. viride*	Meiji Seik Kaisha, Japan
Hemicellulase		
Hemicellulase	*Aspergillus niger*	Sigma, USA
Rhozyme HP-150	*Aspergillus niger*	Rohm and Hass, USA
Zymolyase	*Arthrobacter luteus*	Sigma, USA
Pectinase		
Macerozyme R-10	*Rhizopus* sp.	Yakult Honsha, Japan
Macerase	*Rhizopus* sp.	Calbiochem., USA
Pectinase (purified)	*A. niger*	Sigma, USA
Pectolyase Y23	*A. japonicus*	Seishin Pharmaceutical, Japan
Pectinol	*A. niger*	Rohm and Hass, USA

genic and non-embryogenic suspension cultures (Bhojwani and Razdan, 1983). More recently, viable protoplasts have been obtained from male and female gametes (see Chapter 10).

Isolation of viable and culturable protoplasts in large quantities is affected by several factors, and optimum conditions for a system are established empirically. The work of Uchimiya and Murashige (1974) with cultured cells of tobacco should serve as a model when attempting to isolate protoplasts from a new system (see Table 12.2).

Protocols for isolation of protoplasts from mesophyll cells, seedling explants and cultured cells of some plants are given in Appendix 12.1 (see also Fig. 12.1).

12.2.1. Factors affecting yield and viability of protoplasts

(i) *Source of material.* Leaf has been the most favourite source of plant protoplasts because it allows the isolation of a large number of relatively uniform cells without the necessity of killing the plants. Since the mesophyll cells are loosely arranged, the enzymes have an easy access to the cell wall. When protoplasts are prepared from leaves the age of the plant

TABLE 12.2

Optimal conditions for the isolation of protoplasts from cultured cells of tobacco[a]

Parameter	Optimum condition
Plant material	4–5-day-old subculture
Cellulase	1% Onozuka R-10
Macerozyme	0.1–0.2% Onozuka R-10
pH of enzyme solution	4.7–5.7
Volume of enzyme solution/fresh weight of tissue	10 ml g^{-1}
Incubation period	2–3 h
Incubation temperature	22–37°C
Rate of agitation	50 rev. min^{-1}
Osmoticum	300–800 mmol l^{-1} mannitol

[a]After Uchimiya and Murashige (1974).

and the conditions under which it has been grown may be critical. To achieve maximum control on the growth conditions of source plants several workers have used in vitro growing shoots. Leaves from such plants also do not require exposure to surface sterilants. The leaves from in vitro rooted shoots of *Pyrus communis* released twice as many viable protoplasts as the leaves from field grown material (Ochatt and Caso, 1986). When the leaves are derived from glasshouse- or growth room-grown plants, it would be desirable to optimize the growth conditions (light, humidity, temperature and supply of nutrients) for the donor plants. For *Brassica* species, hypocotyl segments from aseptic seedlings have been widely used to isolate protoplasts (Glimelius, 1984; Chuong et al., 1985; Barsby et al., 1986; Chuong et al., 1987a,b; Yamashita and Shimamoto, 1989).

Owing to the difficulty in isolating culturable protoplasts from leaf cells of cereals and some other species their cultured cells have been used as an alternative source material. The yield of protoplasts from cultured cells depends on the growth rate and growth phase of the cells. Frequently sub-cultured (every 3–7 days) suspension cultures, and cells taken from the early log phase are most suitable. To obtain totipotent protoplasts generally embryogenic suspension cultures are used.

(ii) *Pre-enzyme treatments.* To facilitate the penetration of enzyme solution into the intercellular spaces of leaf, which is essential for effective digestion, various methods are followed. A most commonly practised method is to peel the lower epidermis and float the stripped pieces of leaf on the enzyme solution in a manner that the peeled surface is in contact

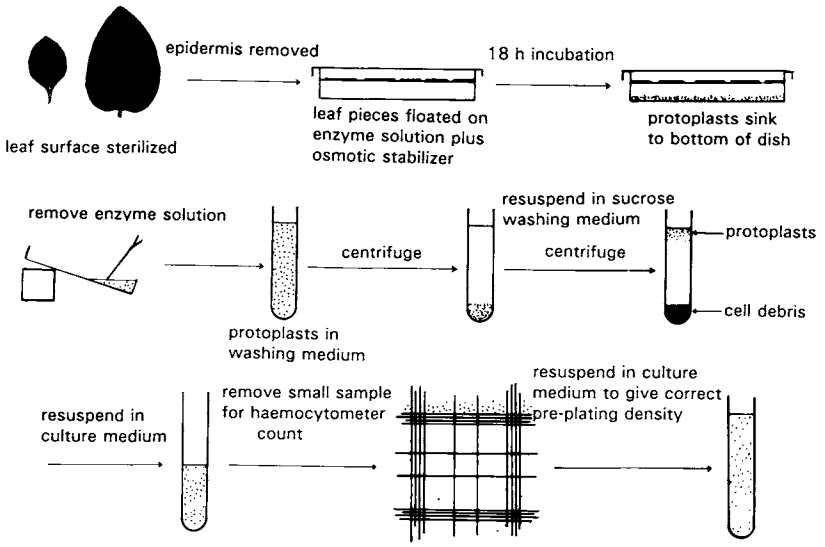

Fig. 12.1. Flow diagram for the isolation of mesophyll protoplasts (courtesy of E.C. Cocking, UK).

with the solution. Where it is not possible or is inconvenient to peel the epidermis, cutting the leaf or tissue into small strips (1–2 mm wide) has been found useful. When combined with vacuum infiltration the latter approach has proved very effective. Mesophyll protoplasts of cereals could be isolated within 2 h by infiltrating the leaf pieces with enzyme solution under a partial vacuum for 3–5 min (Scott et al., 1978). The criterion used to check adequate infiltration is that leaf pieces will sink when the vacuum is removed. Brushing the leaf with a soft brush or with the cutting edge of a scalpel may also improve enzymatic action, and cutinase has been used to remove leaf epidermis (Power et al., 1989). Large calli are chopped into small pieces before transfer to enzyme mixture.

Agitation of the incubation mixture during enzyme treatment improves protoplast yield from cultured cells.

(iii) *Enzyme treatment.* The release of protoplasts is very much dependent on the nature and concentration of the enzymes used. The two enzymes regarded essential to isolate protoplasts from plant cells are cellulase and pectinase. The latter degrades mainly the middle lamella and the former is required to digest the cellulosic cell wall. The first commercially available enzymes of fungal origin were Onozuka Cellulase SS and Onozuka Macerozyme SS. Due to increasing demands for these

enzymes many other companies are now producing these enzymes and marketing under different trade names (see Table 12.1). Driselase, having a number of zymolytic activities, such as cellulase, pectinase, laminarinase and xylanase (Kao et al., 1974), has proved especially useful for isolating protoplasts from cultured cells. Even purified enzymes, like cellulase R-10, seem to carry adequate pectinase (Okuno and Furusawa, 1977; Slabas et al., 1979). Pectolyase Y-23, a highly powerful macerozyme, in combination with cellulase released protoplasts from mesophyll cells of pea within 30 min (Nagata and Ishii, 1979).

Some tissues may also require hemicellulase in addition to cellulase and macerozyme. Aleurone cells of barley treated with cellulase did not liberate protoplasts. A thin cellulase-resistant wall was left around them. Such cells, called spheroplasts, had to be treated with glusulase to digest the remaining wall (Taiz and Jones, 1971).

The crude commercial enzymes carry nucleases and proteases as impurities which may be harmful to protoplasts viability. Therefore, some workers prefer to purify the enzymes by eluting them through biogel or Sephadex G-25 filtration (Constabel, 1982). However, mostly the enzymes have been employed in their crude forms with satisfactory results. Indeed, Arnold and Eriksson (1976) observed that purification of enzymes resulted in fewer surviving protoplasts, and the crude enzymes were more effective.

The activity of enzymes is pH dependent. The optimal pH values of Onozuka cellulase R-10 and macerozyme R-10, as given by the manufacturers, are 5–6 and 4–5, respectively. In practice, however, the pH of the enzyme solution is mostly adjusted anywhere between 4.7 and 6.00.

The optimal temperature for the activity of these enzymes is 40–50°C which happens to be too high for the cells. Generally 25–30°C is found adequate for isolation of protoplasts. The concentration of the enzymes and the duration of enzyme treatment is to be decided after several trials. The incubation period in the enzyme solution may be as short as 30 min (Nagata and Ishii, 1979). Another factor that may affect the yield of protoplasts is the relative volume of the enzyme solution to the amount of tissue. Generally 10 ml solution for 1 g tissue is satisfactory.

The cells that are damaged or lysed during isolation may release hydrolytic enzymes capable of damaging the healthy protoplasts. To counter this problem, addition of potassium dextran sulphate (0.5% w/v) to the enzyme solution has been recommended (Ochatt and Power, 1992). Addition of antioxidants, such as PVP-10 (average MW 10 000) to the enzyme mixture has proved essential for isolation of large numbers of viable protoplasts of recalcitrant plants such as deciduous tree species (Revilla et al., 1987).

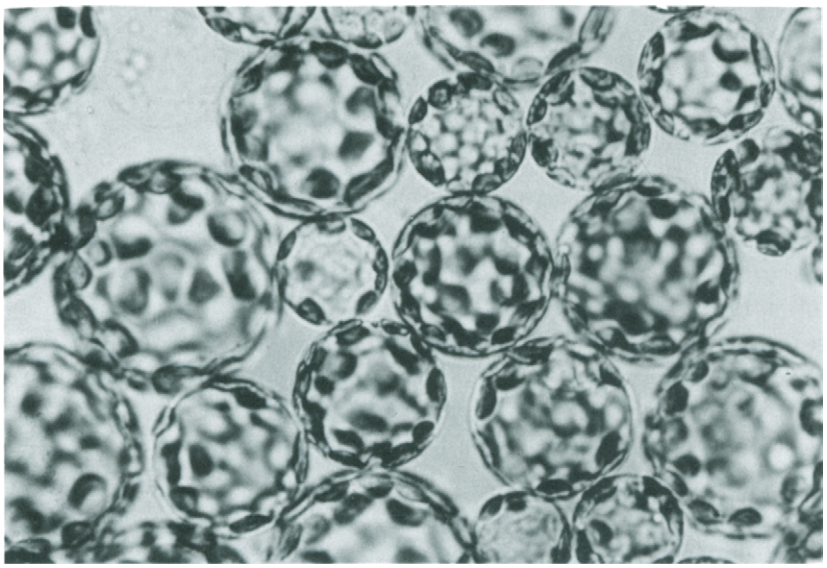

Fig. 12.2. Freshly isolated mesophyll protoplasts (courtesy of J.B. Power, UK).

(iv) *Osmoticum.* A fundamental property of isolated protoplasts is their osmotic fragility and, hence, the need for a suitable osmotic stabilizer in the enzyme solution, the protoplast washing medium, and the protoplast culture medium. In a solution of proper osmolarity freshly isolated protoplasts appear completely spherical (see Figs. 12.2 and 12.4A). On a quantitative basis protoplasts are more stable in a slightly hypertonic rather than isotonic solution. A higher level of the osmoticum may prevent bursting and budding but, at the same time, it may inhibit the division of the protoplasts.

A variety of solutes, ionic and non-ionic, have been used for adjusting the osmotic pressure of the various solutions used in protoplast isolation and culture but the most widely used osmotica are sorbitol and mannitol in the range 450–800 mmol. Uchimiya and Murashige (1974) observed that for isolating protoplasts from tobacco suspension cultures several soluble carbohydrates, including glucose, fructose, galactose, sorbitol and mannitol, were equally effective. When non-ionic substances are used as osmotic stabilizer the enzyme solution is often supplemented with certain salts, especially $CaCl_2$ (50–100 mmol l^{-1}). This improves the stability of the plasma membrane.

Meyer (1974) and Bohnke and Kohlenbach (1978) reported that the use of ionic osmotica (335 mmol l^{-1} KCl and 40 mmol l^{-1} $MgSO_4 \cdot 7H_2O$) im-

proved the viability of the protoplasts and yielded cleaner preparations. However, adjusting the osmotic pressure of the culture medium with salts proved detrimental.

12.2.2. Purification of protoplasts

After the material has been incubated in enzyme solution for an adequate period the incubation vessel is gently swirled or the leaf pieces are gently squeezed to release the protoplasts held in the original tissue. The digestion mixture at this stage would consist of subcellular debris, especially chloroplasts, vascular elements, undigested cells and broken protoplasts, besides intact and healthy protoplasts. It is, therefore, necessary to remove these contaminants. The large debris is removed by passing the digestion mixture through a metal or nylon sieve (30–100 μm pore size). For further purification one of the following two methods has been generally followed. Generally, the filtrate is sedimented in a centrifuge tube at 100 × g for about 5 min, and the supernatant containing small debris is discarded. The pellet is resuspended in the washing medium and washed three times by repeated centrifugation at 50 × g for 3–5 min and resuspension. Alternatively, the pelleted protoplasts and debris suspended in a small volume of the enzyme mixture or the washing medium is loaded at the top of a sucrose pad (21%) in a centrifuge tube and spun at 100 × g for 10 min. The debris moves down to the bottom of the tube and a band of clean protoplasts appears at the junction of the sucrose pad and the protoplast suspension medium. The protoplasts are gently removed with a Pasteur pipette and transferred to another centrifuge tube. Following the repeated centrifugation and resuspension, as in the first procedure, the protoplasts are washed three times and finally suspended in the culture medium at an appropriate density.

Hughes et al. (1978) purified the protoplasts using 450 mmol l^{-1} sucrose (bottom) and 450 mmol l^{-1} mannitol (top) discontinuous gradient. Piwowarczyk (1979) modified the density gradient in a way that intact protoplasts, free of the enzyme and debris, can be obtained by a single spinning. The gradient is prepared by sequentially filling the centrifuge tube with 500 mmolv sucrose in the culture medium, a layer of 140 mmol l^{-1} sucrose and 360 mmol l^{-1} sorbitol in the culture medium and, finally, a layer of protoplast suspension in the enzyme solution which contains 300 mmol l^{-1} sorbitol and 100 mmol l^{-1} $CaCl_2$. After spinning at 400 × g for 5 min a clean layer of protoplasts is formed just above the sucrose layer and the debris moves down to the bottom of the tube. Larkin (1976) and Scowcroft (1977) found the commercial density buffer Lymphoprep (Nyegaard A/S, Oslo, Norway) to be excellent for removing debris. The

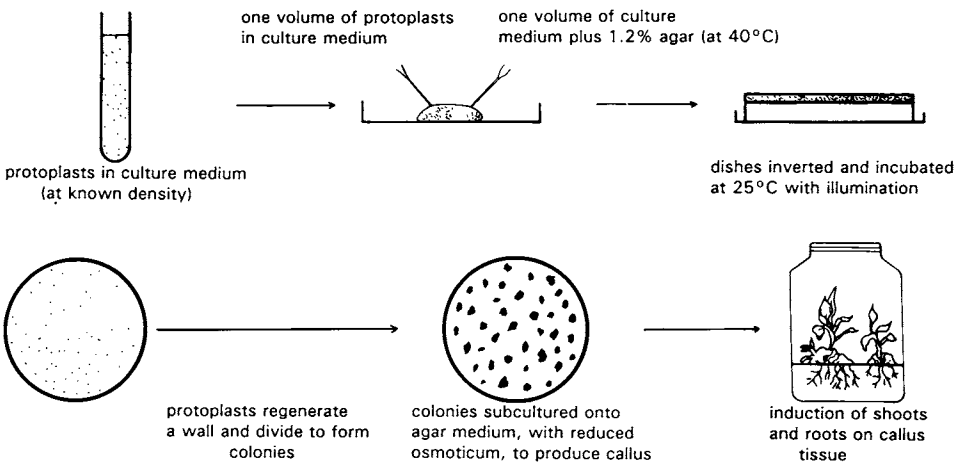

Fig. 12.3. Flow diagram for the culture of protoplasts (courtesy of E.C. Cocking, UK).

buffer comprises 9.6% (w/v) sodium metrizoate and 5.6% (w/v) Ficoll, with a specific gravity of 1.077 ± 0.001 g ml^{-1}.

From previous notes of single cell culture

12.2.3. Viability of the protoplasts

Viability of the freshly isolated protoplasts can be checked by a number of methods: (a) observation of cyclosis or cytoplasmic streaming as an indication of active metabolism. This method is not very helpful with mesophyll protoplasts which carry a large number of peripheral chloroplasts; (b) oxygen uptake measured by an oxygen electrode which indicates respiratory metabolism (Taiz and Jones, 1971); (c) photosynthetic activity (Kanai and Edwards, 1973); (d) exclusion of Evan's blue dye by intact membranes (Kanai and Edwards, 1973; Glimelius et al., 1974); and (e) staining with fluroescein diacetate. The last method is most commonly used. Some of the methods to test cell/protoplast viability are described in Section 4.3.6.

12.3. PROTOPLAST CULTURE

From previous notes of single cell culture

The culture methods and the culture requirements of isolated protoplasts are often similar to those of single cells. Protoplasts may be cultured in agar plates (see Fig. 12.3) following the Bergmann's technique of cell plating (see Chapter 4). An advantage in using semi-solid medium is that the protoplasts remain stationary which makes it convenient to follow the development of specific individuals. However, liquid medium has

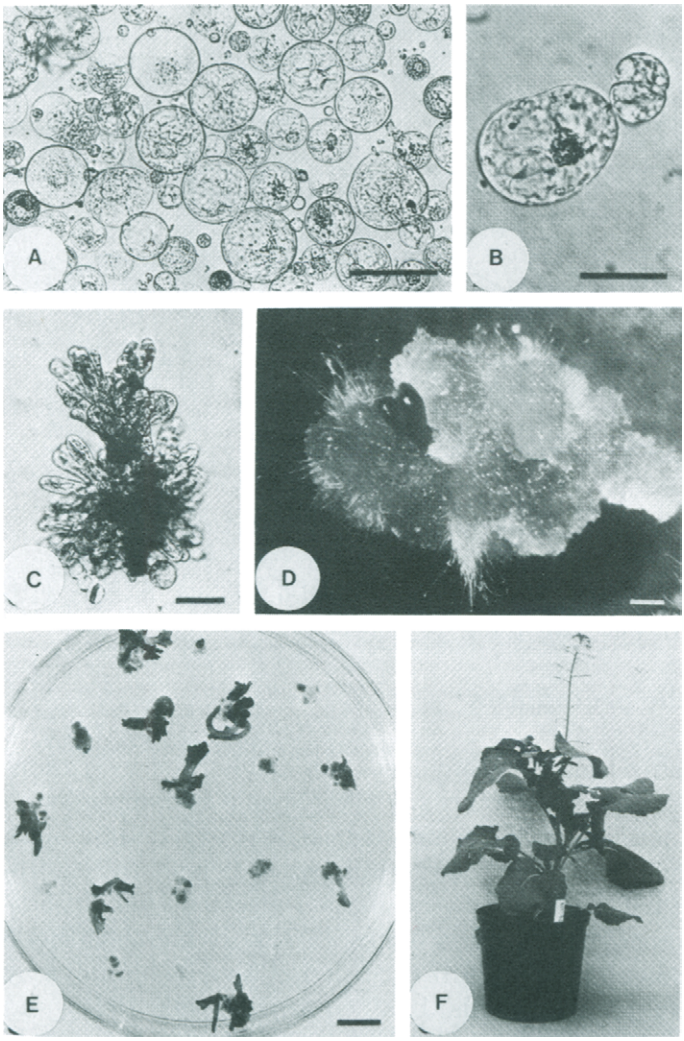

Fig. 12.4. Plant regeneration from hypocotyl protoplasts of *Brassica napus*. (A) Freshly isolated protoplasts of different sizes. (B) One-day-old protoplasts; both the protoplasts have regenerated wall but only the small one has divided. (C) Callus obtained from the protoplasts after 14 days of culture. (D) Differentiation of shoot buds from the protoplast-derived callus. (E) Calli with regenerated shoots as in (D), transferred to regeneration medium containing zeatin and IAA. (F) Plant regeneration from protoplast-derived callus (reprinted by permission from K. Glimelius, 1984, Physiol. Plant., 61: 38–44).

been generally preferred for the following reasons: (a) protoplasts of some species would not divide if plated in agarified medium (Gosch et al., 1975; White and Bhojwani, 1981), (b) the osmotic pressure of the medium can be effectively reduced after a few days of culture, (c) if the degenerating

component of the protoplast population produces some toxic substances which could kill the healthy cells it is possible to change the medium (Schenck and Hoffmann, 1979; Bhojwani and White, 1982), and (d) the density of cells can be reduced or cells of special interest may be isolated after culturing them for a few days at a high density (Kao, 1977; Gleba, 1978; Gleba and Hoffmann, 1978; White and Bhojwani, 1981). In liquid medium protoplasts have been cultured variously. The protoplast suspension is plated as a thin layer in petri plates, incubated as static cultures in flasks (about 5 ml of the protoplast suspension in 50–100 ml flasks) (Takebe and Nagata, 1973; Coutts and Wood, 1975), or distributed in 50–100 μl drops in petri plates and stored in a humidified chamber.

Embedding protoplasts in agarose beads or discs is reported to improve plating and regeneration efficiency in many species (see Dons and Colijn-Hooymans, 1989). The protoplasts of several recalcitrant species of Magnoliaceae and Liliaceae divided and regenerated when their protoplasts were trapped in agarose droplets in such a way that streaks of locally high cell densities were obtained (Binding et al., 1988). In practice, the protoplasts suspended in molten (40°C) agarose medium (1.2% w/v agarose) are dispensed (4 ml) into small (3.5–5 cm diameter) plates and allowed to solidify. The agarose layer is then cut into 4 equal sized blocks and transferred to larger dishes (9 cm diameter) containing liquid medium of otherwise the same composition (Shillito et al., 1983). Alternatively, protoplasts in molten agarose medium are dispensed as droplets (50–150 μl) on the bottom of petri plates and after solidification the droplets are submerged in the same liquid medium.

Alginate is another gelling agent used for culture of protoplasts, particularly of the species which are heat sensitive, such as *Arabidopsis thaliana* (Damm and Willmitzer, 1988).

Even a healthy protoplast preparation, under the most favourable culture conditions, shows bursting of some protoplasts during the first 24 h in culture. The stable protoplasts rapidly resume recovery from the trauma of being stripped off their wall. They exhibit an active increase in the number of cell organelles, cytoplasmic streaming, respiration, and synthesis of RNA, protein, and polysaccharides, suggesting an active cellular metabolism.

12.3.1. Cell wall formation

Within 2–4 days in culture, protoplasts lose their characteristic spherical shape and this has been taken as an indication of new wall regeneration. More reliable and direct demonstration of wall regeneration has been through staining with Calcofluor White ST (American Cyanamide

Co., Wayne, NJ, USA)[2] and from the use of a variety of electron microscopic techniques (Willison, 1976; Fowke, 1978).

The regularity of cell wall regeneration and the lag period prior to the onset of wall formation depends partly on the plant species and the degree of differentiation of the cells used for protoplast isolation (Ochatt and Power, 1992). Mesophyll, callus and cell suspension protoplasts of most solanaceous and many *Brassica* species form cell wall very quickly (within 24–40 h of culture) (Gamborg et al., 1981; Evans and Bravo, 1983; Power et al., 1989). In contrast, cereal protoplasts (Vasil, 1987, 1988) and mesophyll protoplasts of legumes (Davey and Power, 1988) may require up to 4 days for cell wall regeneration. An even longer lag phase (7 days or more) is required for wall formation by the protoplasts of the woody plants (McCown and Russell, 1987; Vardi and Galun, 1988).

Horine and Ruesink (1972) reported that the cell wall regeneration in *Convolvulus* protoplasts required an exogenous supply of a readily metabolizable carbon source, such as sucrose. In its absence cell wall formation did not occur. Ionic osmotic stabilizers in the culture medium suppress the development of a proper wall (Meyer, 1974; Meyer and Abel, 1975; Bohnke and Kohlenbach, 1978). The protoplasts of carrot cell suspensions developed a wall faster and more uniformly if polyethylene glycol 1500 was added to the culture medium (Wallin and Eriksson, 1973).

There is a direct relationship between wall formation and cell division. Protoplasts which are not able to regenerate a proper wall fail to undergo normal mitosis (Bawa and Torrey, 1971; Meyer and Abel, 1975; Bohnke and Kohlenbach, 1978). Protoplasts with a poorly developed wall often show budding and may enlarge several times their original volume. They may become multinucleate because karyokinesis is not accompanied by cytokinesis. Among other reasons, inadequate washing of the protoplasts prior to culture leads to these abnormalities.

12.3.2. Cell division and callus formation

While the presence of a proper wall is essential for regular division, not all such cells regenerated from protoplasts embark upon division. The

[2] To test the presence of a wall, protoplasts are incubated in 0.01 or 0.1% Calcofluor solution, in an appropriate osmotic stabilizer, for 5 min. The protoplasts are then washed to remove any excess dye and mounted on a slide, in an osmotically suitable solution. Calcafluor binds to the wall material and fluoresces when observed using a mercury vapour lamp, with an excitation filter BG12, and suppression filter K 510. Tinapol Solution B.O.P.T (Geigy U.K. Ltd., Dye Stuff and Textile Chemicals Div. Simonsway, Manchester, UK) behaves in a similar way to Calcafluor (Evans and Cocking, 1975).

plating efficiency of protoplasts varies considerably with the experimental material; it may range from as low as 0.1% to as high as 80%.

The protoplasts capable of dividing, undergo the first division within 2–7 days (see Fig. 12.4B). Rarely, the lag phase before the first division lasts as long as 7–25 days (Bhojwani et al., 1977b; Khasanov and Butenko, 1979; Ochatt, 1990). Protoplasts from actively dividing cell suspensions, as a rule, enter the first division faster than those from highly differentiated cells of the leaf. The cells which continue dividing develop multicellular colonies after 2–3 weeks in culture (see Fig 12.4C). After another 2 weeks macroscopic colonies are formed which can be transferred to osmoticum-free medium and treated as standard tissue cultures (see Fig. 12.4D–F).

In protoplast cultures, the cell divisions are asynchronous. The first division may be equal or unequal. Mitosis is normal. Several factors influence divisions in protoplast cultures.

(i) *Nutritional requirements*. Mostly the salts of MS (Murashige and Skoog, 1962) and B_5 (Gamborg et al., 1968) media and their modifications have been used. Kao et al. (1973) reported that the addition of 1 mmol l^{-1} $CaCl_2$ to B_5 medium improved the percentage of dividing cells in protoplast cultures of *Vicia hajastana* and *Bromus inermis*. However, supplementing the medium with 20 mmol l^{-1} NH_4NO_3 reduced the frequency of dividing cells. Ammonium ions proved detrimental to protoplast survival of many other species, and media have been devised that either have a reduced concentration of ammonium (paper mulberry, Oka and Ohyama, 1985) or lack it (potato, Upadhya, 1975; tomato, Zapata et al., 1981; tobacco, Caboche, 1980; *Pyrus*, Ochatt and Caso, 1986; Ochatt and Power, 1988a,b).

Vitamins used for protoplast culture are the same as those used in standard tissue culture media. The 8p medium developed by Kao and Michayluk (1975) for low density protoplast culture contains several vitamins, organic nutrients, sugar alcohols and undefined nutrients such as casamino acids and coconut water (Table 12.3). This medium and modifications thereof have been used successfully on a broad range of species including cereals (Thompson et al., 1986b), legumes (Gilmour et al., 1987), ornamentals (Power et al., 1989) and fruit trees (Patat-Ochatt et al., 1988).

Growth hormones, particularly auxins and cytokinins, are almost always required. For cereal protoplasts, however, 2,4-D alone is either sufficient or better than in combination with cytokinin. The type of auxin and cytokinin and their ratios in the medium required to induce divisions at optimum rate may vary considerably with the plant material. The

TABLE 12.3

A medium for culturing protoplasts at low density[a,b]

Constituents	Amount (mg l^{-1})	Constituents	Amount (mg l^{-1})
Mineral salts			
NH_4NO_3	600	KI	0.75
KNO_3	1900	H_3BO_3	3.00
$CaCl_2 \cdot 2H_2O$	600	$MnSO_4 \cdot H_2O$	10.00
$MgSO_4 \cdot 7H_2O$	300	$ZnSO_4 \cdot 7H_2O$	2.00
KH_2PO_4	170	$Na_2MoO_4 \cdot 2H_2O$	0.25
KCl	300	$CuSO_4 \cdot 5H_2O$	0.025
Sequestrene 330 Fe[c]	28	$CoCl_2 \cdot 6H_2O$	0.025
Sugars			
Glucose	68400	Mannose	125
Sucrose	125	Rhamnose	125
Fructose	125	Cellobiose	125
Ribose	125	Sorbitol	125
Xylose	125	Mannitol	125
Organic acids (adjusted to pH 5.5 with NH_4OH)			
Sodium pyruvate	5	Malic acid	10
Citric acid	10	Fumaric acid	10
Vitamins			
Inositol	100	Biotin	0.005
Nicotinamide	1	Choline chloride	0.5
Pyridoxine·HCl	1	Riboflavin	0.1
Thiamine·HCl	10	Ascorbic acid	1
D-Calcium pantothenate	0.5	Vitamin A	0.005
Folic acid	0.2	Vitamin D_3	0.005
p-Aminobenzoic acid	0.01	Vitamin B_{12}	0.01
Hormones	Soybean × barley	Soybean × pea or *N. glauca*	
2,4-D	1	0.2	
Zeatin	0.1	0.5	
NAA	–	1	
Vitamin-free casamino acid[d]	125 mg l^{-1}		
Coconut water (from mature fruits; heated to 60°C for 30 min and filtered)	10 ml l^{-1}		

[a]After Kao and Wetter (1977).
[b]Sterilized by filtration.
[c]Geigy Chemical Corp., Ardsley, NY.
[d]Difco Laboratories, Detroit, MI.

most commonly used auxin is 2,4-D, but Uchimiya and Murashige (1976) reported that NAA was superior to 2,4-D or IAA for the culture of protoplasts from cell suspensions of tobacco. The cytokinins commonly used are BAP, kinetin and 2-ip. Whereas the protoplasts from actively growing cultured cells may find a high auxin/kinetin ratio suitable for their division, those derived from highly differentiated cells, such as leaf cells, often require a high kinetin/auxin ratio for dedifferentiation.

Sometimes culture requirements of intact cells and tissues may give clues to the composition of the medium suitable for their protoplast culture, but the simple concept of the cultural behaviour of protoplasts being equivalent to that of cells without a cell wall is not always valid. For example, the growth regulator autonomy of cultured-crown gall tumour cells is lost upon removing the cell wall and is restored at the multicellular stage (Scowcroft et al., 1973). Similarly, culture requirements of pea shoot-tip protoplasts are different from those of its cells (Gamborg et al., 1975). Scott et al. (1978) observed that freshly isolated protoplasts of cereals were sensitive to phytohormones in the medium but cells regenerated from them could be transferred to a medium containing auxin and cytokinin to induce divisions.

The low viability of protoplasts of *Lycopersicon pennellii* could be correlated with high ethylene production and increased cell osmolality (Rethmeier et al., 1991). Cell wall degrading enzymes also influence the release of ethylene. Addition of ethylene-inhibitor, silver thiosulphate to the culture medium improved yield, viability and regeneration of protoplasts of *L. pennellii* (Rethmeier et al., 1991) and potato (Perl et al., 1988; Mollers et al., 1992).

Antioxidants in the medium are either essential or improve the response of cultured protoplasts in some cases. For protoplast culture of sweet cherry (*Prunus avium*) addition of the antioxidants, glycine and/or PVP-10, to the culture medium was essential to counter the phenolic browning of protoplasts and protoplast derived tissues at all culture stages (Ochatt, 1991). In *Beta vulgaris* addition of the antioxidant n-propylgallate (n-PG) to the medium proved essential for successful culture of protoplasts and shoot regeneration (Krens et al., 1990). A combination of the antioxidants glutathione, glutathione-peroxidase and phospholipase increased the plating efficiency and growth of microcalli from protoplasts of *Lolium perenne* (Creemers-Molenaar and Van Oort, 1990).

Addition of 2% Ficoll to the culture medium more than doubled the cell division frequency in mesophyll protoplast cultures of *Brassica napus* (Millam et al., 1988). The colonies on this medium were larger and greener than those on the control, probably because of better aeration of

the cells; in the presence of Ficoll protoplasts and microcolonies float on the medium.

(ii) *Osmoticum*. Isolated protoplasts require osmotic protection in the culture medium until they regenerate a strong wall. Osmolarity of the medium is generally adjusted with 500–600 mmol l^{-1} mannitol or sorbitol, as in the enzyme solution. Scott et al. (1978) and Arnold and Eriksson (1976) reported that for mesophyll protoplasts of cereals and pea, respectively, sucrose or glucose could not replace mannitol or sorbitol as the osmotic stabilizer in the medium. However, some authors have noted the superiority of glucose over other osmotic agents (Gamborg et al., 1975; Michayluk and Kao, 1975; Evans et al., 1980). Shepard and co-workers (Shepard and Totten, 1977; Bidney and Shepard, 1980; Shahin and Shepard, 1980) routinely used sucrose as the osmotic stabilizer for the culture of protoplasts of potato, sweet potato, and cassava. For brome grass, sucrose proved better than glucose or mannitol (Michayluk and Kao, 1975). The use of an ionic osmoticum in the culture medium suppresses the regeneration of a proper wall, leading to the failure of normal mitosis (Horine and Ruesink, 1972; Meyer, 1974; Meyer and Abel, 1975; Bohnke and Kohlenbach, 1978).

Seven to ten days after initial culture, by which time most of the viable protoplasts have regenerated a good wall and undergone a few divisions, the osmolarity of the medium is gradually reduced by periodic addition of a few drops of fresh medium lacking in the osmoticum or containing it at a fairly low level. In the presence of the original high level of the osmoticum the cells may stop dividing after some time (Kao and Michayluk, 1980). Macroscopic colonies are finally transferred to a fresh medium lacking the osmoticum.

(iii) *Plating density*. As in cell cultures, the initial plating density of protoplasts has a profound effect on plating efficiency. Protoplasts are generally cultured at a density of 1×10^4 to 1×10^5 protoplasts ml^{-1} of the medium. At such high densities the cell colonies arising from individual protoplasts tend to grow into each other at a fairly early stage in culture. This would result in the formation of chimeral tissue if the protoplast population was genetically heterogeneous. Cloning of individual cells, which is highly desirable in somatic hybridization and mutagenic studies, can be achieved if protoplasts or cells derived from them can be cultured at a low density (100–500 protoplasts ml^{-1}). It may also allow the development of individual cells to be followed, thus enabling the solation of hybrid colonies in the absence of a stringent selection system.

Kao and Michayluk (1975) developed a complex culture medium (see Table 12.3) in which individually cultured protoplasts of *Vicia hajastana* regenerated a wall, underwent sustained divisions and formed callus. In this medium (8p) mesophyll protoplasts of alfalfa, pea, and *Vicia* divide faster at lower population densities (less than 100 protoplasts ml^{-1}) than at higher densities. The 8p medium and its various modifications have been successfully used to culture protoplasts of a range of other species. While using 8p medium the cultures should be stored in the dark or under very low light intensity (50 lx) because in strong light the medium becomes phytotoxic (Kao and Wetter, 1977).

The feeder cell layer technique, developed by Raveh et al. (1973) for plant protoplasts, is another approach to culture protoplasts at low densities. Tobacco protoplasts normally do not divide at a plating density below 10^4 protoplasts ml^{-1} but with the feeder cell layer they could be cultured at a density as low as 10–100 protoplasts ml^{-1} (Raveh et al., 1973; Raveh and Galun, 1975). The feeder cell layer was prepared by exposing the protoplasts (10^6 cells ml^{-1}) to an X-ray dose of 5×10^3 R which inhibited cells from dividing but allowed them to remain metabolically active. Protoplasts were then washed two to three times (it is important to wash properly to remove any toxic substances produced due to irradiation) and plated in soft agar. Non-irradiated protoplasts in agar medium were layered over the feeder cell layer. The optimal density of cells in the feeder layer was the same as the optimal plating density of the protoplasts cultured without the feeder layer (2.4×10^4 ml^{-1}). The feeder layer can also be prepared with cells from suspension cultures (Cella and Galun, 1980).

The importance of the feeder layer or nurse culture has now been demonstrated for various crops (Jain et al., 1995). Individual protoplasts of barley, tobacco and rape could be successfully cultured using feeder systems (Eigel and Koop, 1989; Schaffler and Koop, 1990). A simple versatile feeder layer system for *Brassica oleracea* protoplasts has been described by Walters and Earle (1990). Kyozuka et al. (1987) used actively growing nurse cells in liquid medium to support the regeneration of rice protoplasts embedded in agarose beads (Fig. 12.5). Jain et al. (1995) have shown that feeder layers with *Oryza ridleyi* or *Lolium multiforum* were able to induce division in protoplasts of two indica rice varieties which did not divide otherwise. The latter was four times more effective than the former. Feeder layers with cells of both the species gave maximum plating efficiency.

Some cross-feeding is known to occur between protoplasts of different species (Vardi, 1978; Butenko and Kuchko, 1980; Cella and Galun, 1980). However, for tobacco and orange protoplasts the feeder layer prepared with their own protoplasts was more effective than that with alien cells

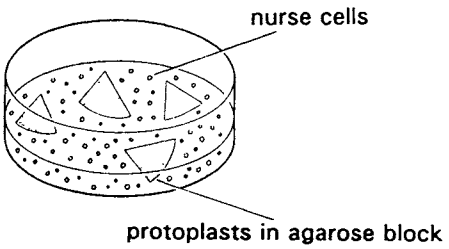

Fig. 12.5. Nurse culture of rice protoplasts. The protoplasts are embedded in nutrient agarose blocks and suspended in liquid medium containing nurse cells (reprinted by permission from: J. Kyozuka et al., 1987, Mol. Gen. Genet., 206: 408–413; © Springer-Verlag).

(Vardi, 1978). Similarly, in oat feeders from graminaceous plants promoted protoplast proliferation while feeders from dicotyledonous species suppressed protoplast division (Hahne et al., 1990).

The microdroplet technique was used by Kao (1977), Gleba (1978) and Gleba and Hoffmann (1978) to culture individual protoplasts and cells regenerated from them. They used special Cuprak dishes which have two chambers, a small outer chamber and a large inner chamber. The latter carries numerous numbered wells each with a capacity of 0.25–25 μl of nutrient medium. The protoplast suspension is transferred to the wells as microdroplets. The outer chamber is filled with sterile distilled water to maintain the humidity inside the dish. After covering it with the lid the dish is sealed with parafilm. Following this method, Gleba (1978) obtained whole plants of tobacco from protoplasts cultured individually in 0.25–0.5 μl droplets. The size of the droplets is critical for the division of single protoplasts. One protoplast per 0.25–0.5 μl droplet gives a ratio of cell/volume of culture medium equal to a cell density of 2–4 $\times$ 10^3 ml^{-1}. An increase in the size of the droplet would decrease the effective plating density. Gleba (1978) reported that droplets larger than 2 μl did not support the division of individual cells. The microdroplet method has been successfully used to culture hybrid cells of *Nicotiana glauca* + *Glycine max* (Kao, 1977) and *Arabidopsis thaliana* + *Brassica campestris* (Gleba and Hoffmann, 1978).

Koop and Schweiger (1985a) described a microculture system based on a computer controlled set-up for the efficient selection, transfer and culture of isolated single protoplasts of tobacco in microdroplets (ca. 50 nl) of fully defined medium. Each microdroplet is contained within a separate drop (1 μl) of mineral oil and 50 such droplets are placed on a coverglass (see Fig. 12.6). For culture, the coverglass is kept in a moist chamber. Subsequently, this technique was successfully applied for plant regeneration from hypocotyl protoplasts of *Brassica napus* (Spangenberg

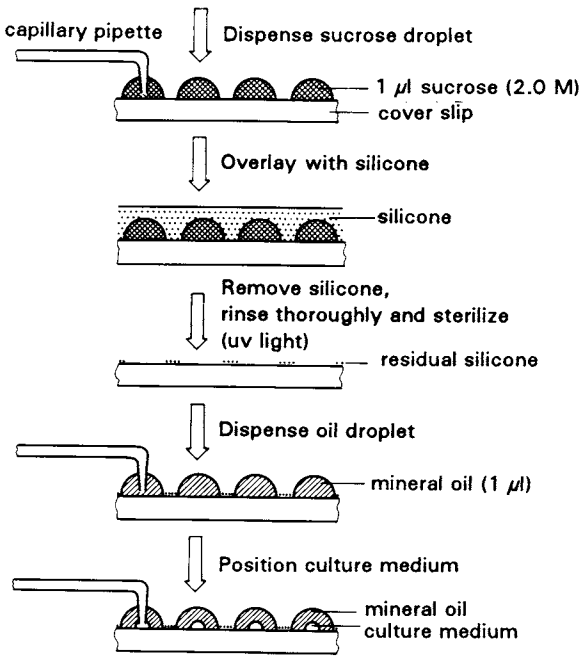

Fig. 12.6. Diagrammatic summary of the procedure followed to prepare a microchamber to culture individual protoplasts, using a 24 × 40 mm coverglass. Fifty 1.0 μl droplets of 2 M sucrose are applied onto the coverglass in an array of 5 rows of 10 drops each. The coverglass carrying the drops is covered with 'Repel silance (solution of dimethyldichlorosilance in 1,1,1-trichloroethane; LKB), drained, washed with water, rinsed with ethanol, dried and UV sterilized. One microlitre of mineral oil droplet is pipetted onto the spot that was occupied by sucrose and injected with 15–100 nl culture medium. The coverglass so prepared is kept in a two-compartment petri plate, with the outer compartment filled with 2 ml of 0.2 M mannitol solution (reprinted by permission from: H.U. Koop and H.G. Schweiger, 1985, J. Plant Physiol., 121: 245–257; © Gustav Fischer Verlag).

et al., 1985, 1986). A summarized description of the experimental set-up and protocol to be followed has been described by Schweiger et al. (1987).

Culturing the protoplasts at a high density for 4–5 days and then transferring to a fresh medium at very low densities (1–15 cells ml^{-1}) has been reported by Caboche (1980) and White and Bhojwani (1981). This two-step procedure would be useful in somatic hybridization if the hybrid cell lines can be distinguished from the parent types at the time of dilution.

(iv) *Physical treatments.* Electroporation treatment of isolated protoplasts has been shown to trigger early onset of cell divisions and bring

about a significant increase of protoplast plating efficiency in herbaceous and woody species (Rech et al., 1987; Chand et al., 1988; Ochatt and Power, 1992; Gupta et al., 1988). The protoplasts suspended in a buffer solution at four times their final density required for culture are exposed to three successive high voltage (250–2000 V) DC pulses, each of 10–50 μs, at intervals of 10 s. The effect of electric treatment of protoplasts seems to be lasting. The calli recovered from electroporated protoplasts of colt cherry showed increased proliferation ability and higher frequency shoot bud differentiation (Chand et al., 1988; Ochatt et al., 1988a; Ochatt, 1990) and such shoots produced a more prolific root system (9–11 roots per shoot) than the untreated controls (Ochatt et al., 1988b). Electroporation of parent protoplasts markedly increased the throughput of heterokaryons following fusion of protoplasts of *Prunus avium* × *pseudocerasus* and *Pyrus communis* (Ochatt et al., 1989). Ochatt and Power (1992) have suggested that the promotory effect of electroportion could be due to enhancement of DNA synthesis which could in turn bring about an earlier expression of genes controlling the early stages of differentiation. This may be coupled with permanent membrane modifications leading to sustained capacity for a longer/more efficient uptake of the requisite media components.

In contrast to the above examples of stimulation of cell division and regeneration in protoplast cultures by electroportion, involving high voltage DC currents, in *Medicago sativa* (Dijak and Simmonds, 1988) and *Trifolium subterraneum* (Zhongyi et al., 1990) low voltage electric treatment of protoplasts is reported to enhance division of protoplasts. Promotion of embryogenic differentiation by low voltage electric treatment is described in Section 6.3.7.

In rice, heat-shock prior to plating the protoplasts doubled the number of protoplasts entering division as well as increased the plating efficiency (Thompson et al., 1987). Gupta et al. (1988) observed stimulation of division and colony formation in protoplast cultures of *Pennisetum squamulatum* by electroporation or heat shock treatment (45°C for 5 min, followed by 10 s on ice). Chilling freshly isolated mesophyll protoplasts of tomato enhanced the plating efficiency by more than twofold (Muhlbach and Thiele, 1981).

(v) *Storage conditions*. Freshly isolated protoplasts should be stored in diffuse light or dark. In some species protoplasts are very sensitive to light and require storage in complete darkness for the first 4–7 days (Krishnamurthi, 1976; Landgren, 1976; Scott et al., 1978). Pea-root protoplasts exposed to 5 min of green filter incandescent light on the stage of the microscope resulted in incomplete inhibition of mitotic activity

(Landgren, 1976). With the regeneration of a proper wall after 5–7 days the cells may become light tolerant and at this stage the cultures may be transferred to light. It has been suggested that in cases where protoplasts are light sensitive observations should be kept to a minimum and those observed should be discarded from subsequent accountability (Torrey and Landgren, 1977). Protoplast cultures are generally maintained at 25–30°C. Very little attention has been paid to the effect of temperature on wall regeneration and subsequent division in protoplast cultures. Mesophyll protoplasts of *Lycopersicon esculentum* and *L. peruvianum* (Zapata et al., 1977) and cultured cell protoplasts of *Gossypium hirsutum* (Bhojwani et al., 1977b) either fail to divide or do so at a very low frequency when stored at 25°C but at 27–29°C they divide with a high plating efficiency. It has been suggested that elevated temperatures may not only influence the rate of division but may also be a pre-requisite for the initiation and maintenance of division in hitherto non-dividing protoplast systems.

(vi) *Plant material.* For reproducibly high plating efficiency the physiological state of the source tissue and the quality of the protoplasts are as critical as, if not more important than, the culture conditions. Therefore, when using tissues from intact plant organs the source plants should be grown under controlled conditions of light, temperature, and humidity. Leaves from field-grown plants often give erratic results. In *Lycopersicon* spp., reduction of the day length from 16 to 9 h and a cold treatment (at 4°C) of the donor plants significantly increased the plating efficiency (Tabaeizadeh et al., 1984).

To overcome the problem of low reproducibility of protocols for protoplast isolation, culture and regeneration, in many species axenic cultures of shoots, maintained under highly controlled environmental conditions are now widely used as the donor tissue. Schenck and Hoffmann (1979) reported that mesophyll protoplasts of *Brassica campestris* and *B. oleracea* isolated from plants grown in a glasshouse or growth chamber failed to divide but those from aseptically growing shoots formed calli. Axenic shoot cultures are also valuable in providing juvenile material of woody species (McCown and Russell, 1987; Revilla et al., 1987; Ochatt, 1990).

The culture response of protoplasts is also affected by the part of aseptic seedlings from which they are derived. In *Helianthus annuus* the hypocotyl-derived protoplasts divided, formed microcalli and, subsequently, somatic embryos but those from cotyledons or leaflets did not even divide (Dupuis et al., 1990). The plating efficiency and the frequency of somatic embryogenesis varied with the portion of hypocotyl from where the pro-

toplasts were obtained. Those from the basal part were most regenerative. Protoplast source can also influence the type of regeneration. Whereas the root protoplasts of *Medicago sativa* showed direct embryogenesis those from leaves or hypocotyl-derived suspension cultures initially formed callus on which somatic embryos could be induced (Pezzotti et al., 1984).

Sometimes when protoplasts from freshly harvested leaves did not divide it has been possible to obtain protoplasts capable of dividing by preculturing the leaves in a suitable medium for 3–7 days (Gatenby and Cocking, 1977; Donn, 1978; Kao and Michayluk, 1980).

In spite of intensive attempts during the past three decades it is still not possible to induce sustained cell divisions in mesophyll protoplasts of graminaceous species (Cutler et al., 1991; Vasil and Vasil, 1991); the recent report of regeneration of plants from mesophyll protoplasts of rice being an exception (Gupta and Pattnayak, 1993). For these plants embryogenic suspension cultures have proved to be the most suitable source of dividing and totipotent protoplasts, and plants have been obtained from protoplasts of almost all graminaceous species for which regenerable suspension cultures have been established.

12.3.3. Plant regeneration

The first report of plant regeneration from isolated protoplasts, in *Nicotiana tabacum* was published in 1971 by Takebe et al. Until 1983 this was achieved with only 44 species, which included 31 solanaceous species (Bhojwani and Razdan, 1983). During 1985–1993 protoplast technology gained considerable momentum and a large number of taxonomically diverse species, including most of the cereals and grasses, several legumes, cotton and tree species, have been shown to regenerate full plants from isolated protoplasts (Fig. 12.4). According to Roest and Gilissen (1993) this has been achieved in 330 species of higher plants (10 species of gymnosperms, 32 monocotyledons and 288 dicotyledons; see Table 12.4).

The main factors responsible for this grand progress are the selection of genotype and choice of source tissue. Plant regeneration in cereals is largely restricted to the protoplasts from embryogenic suspension cultures. Maltose (1.5%) in combination with sucrose (1.5%) substantially improved the regeneration rate from protoplast-derived calli of two indica rice varieties (Jain et al., 1995). In many cases protoplast regeneration appears to be strongly dependent on the genotype. In *Trifolium repens* inter (Yamada, 1989) and intra-varietal (Bhojwani et al., 1984a) variation for regeneration from protoplasts has been reported. Kyozuka et al.

TABLE 12.4

Some examples where whole plants, shoots or embryos have been regenerated from isolated protoplasts[a]

Taxon	Donor tissue	Mode of regeneration	References
Gymnosperms			
Pinaceae			
Abies alba	EC	E	Lang and Kohlenbach (1989)
Larix decidua	EC	E	Von Aderkas (1992)
L. xeurolepis	EC; ES	EP	Klimaszewska (1989)
Picea abies	ES	EP	Gupta et al. (1990)
P. mariana	ES	E	Tautorus et al. (1990)
P. glauca	ES	E	Attree et al. (1987)
Pinus caribaea	ES	E	Laine and David (1990)
P. kesiya	EC	E	Kumar and Tandon (1991)
P. taeda	ES	EP	Gupta and Durzan (1987)
Pseudotsuga menziensii	ES	E	Gupta et al. (1988)
Angiosperms			
MONOCOTYLEDONS			
Amaryllidaceae			
Hemerocallis fulva	P	E	Zhou (1989)
Hemerocallis cv.	SC	P	Fitter and Krikorian (1981)
Araceae			
Caladium bicolor	L	E, S-P	Jing and Wang (1991)
Iridaceae			
Crocus sativus	SC	SP	Isa et al. (1990)
Liliaceae			
Allium cepa	L	B, P	Wang et al. (1986)
Asparagus officinalis	St	Sh	Bui-Dang-Ha and Mackenzie (1973)
Lilium formolongi	EC	EP	Mii et al. (1991a)
Orchidaceae			
Phalaenopsis	C	ShP	Kobayashi et al. (1993)
Poaceae			
Agrostis alba	ES	EP	Asano and Sugiura (1990)
A. palustris	ES	SP	Terakawa et al. (1992)
Festuca rubra	ES	EP	Zaghmout and Terello (1990)
F. arundinacea	ES	EP	Dalton (1988a,b)
Hordeum vulgare	ES	P	Luhrs and Lorz (1988)
Lolium perenne	ES	P	Creemers-Molenaar et al. (1988),
	SC	EP	Dalton (1988a,b)
L. multiflorum	ES	SP	Dalton (1988b)
Oryza refipogon	ES	EP	Baset et al. (1993)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
O. sativa	SC	EP	Abdhullah et al. (1986)
	C	SP	Coulibaly and Demarly (1986),
	SC	P	Fujimura et al. (1985)
	C	P	Hayashi et al. (1986),
	C, SC	EP	Kyozuka et al. (1987),
	SC	EP	Thompson et al. (1986b),
	SC	P	Toriyama et al. (1986),
	SC	SP	Yamada et al. (1986),
Panicum maximum	ES	EP	Lu et al. (1981)
P. miliaceum	SC	EP	Heyser (1984)
Paspalum dilatatum	ES	EP	Akashi and Adachi (1992)
P. scrobiculatum	ES	EP	Nayak and Sen (1991)
Pennisetum americanum	ES	EP	Vasil and Vasil (1980)
P. purpureum	ES	EP	Vasil et al. (1983)
Poa pratensis	SC	Stp	Van der Valk and Zaal (1988)
Saccharum officinarum	SC	SP	Chen et al. (1988)
	ES	EP	Srinivassan and Vasil (1985)
Setaria italica	EC	EP	Dong and Xia (1989)
Sorghum vulgare	ES	SP	Wei and Xu (1990)
Triticum aestivum	ES	EP	Harris et al. (1988)
Zea mays	EC	EP	Cai et al. (1988),
	ES	EP	Kamo et al. (1987),
	ES	EP	Rhodes et al. (1988),
	ES	E	Vasil and Vasil (1987)
DICOTYLEDONS			
Actinidiaceae			
Actinidia chinensis	C	SP	Cai (1988)
A. deliciosa	L, St	P	Cai et al. (1991)
	C, L	E, SP	Oliviera and Pais (1991, 1992)
Apiaceae			
Daucus carota	SC	P	Grambow et al. (1972)
	R	EP	Kameya and Uchimiya (1972)
Foeniculum vulgare	SC	P	Miura and Tabata (1986)
Apocynaceae			
Rauvolfia vomitoria	L	E, ShP	Tremouillaux-Guiller and Chenieux (1991)
Araliaceae			
Panax ginseng	E	EP	Arya et al. (1991)
Asteraceae			
Brachycome dichromosomatica	C	ShP	Hahne and Hoffmann (1986)
Chrysanthemum hortorum	L	SP	Sauvadet et al. (1990)

C. morifolium	L	P	Otsuka (1986), Otsuka et al. (1985)
Cichorium intybus × *endivia*	L	EP	Sidikou-Seyni et al. (1992)
Helianthus annuus	L	ShP	Binding et al. (1980, 1981)
Lactuca saligna	L	ShP	Brown et al. (1987)
L. sativa	L, Co, R	ShP	Berry et al. (1982)
Scenecio fuchsii	Sh	ShP	Binding et al. (1992)
Brassicaceae			
Arabidopsis thaliana	L	SP	Binding et al. (1981)
	SC	ShP	Xuan and Menczel (1980)
Brassica alboglabra	L, Co, R, St	ShP	Pua (1987)
B. campestris	Co	ShP	Glimelius (1984)
B. carinata	Co	ShP	Chuong et al. (1987b)
B. juncea	L	EP	Chatterjee et al. (1985)
B. napus	L	ShP	Kartha et al. (1974),
	R	ShP	Xu et al. (1982b, 1985)
B. nigra	St	ShP	Chuong et al. (1987a),
	SC	E	Klimaszewska and Keller (1986)
B. oleracea	Co	ShP	Vatsya and Bhaskaran (1982)
	R	ShP	Xu et al. (1982b, 1985)
B. rapa	Co	Sh	Hegazi and Matsubara (1992)
Capsella bursa-pastoris	SC	EP	Bonfils et al. (1992)
Diplotaxis muralis	L	E, ShP	Sikdar et al. (1990)
Eruca sativa	L	EP	Sikdar et al. (1987)
Moricandia arvensis	L	SP	Murata and Mathias (1992)
Raphanus sativus	Co	ShP	Hegazi and Matsubara (1992)
Caricaceae			
Carica papaya × *cauliflora*	SC	EP	Chen and Chen (1992)
Caprifoliaceae			
Lonicera nitida	L	SP	Ochatt (1991a)
Caryophyllaceae			
Dianthus barbatus	L	SP	Nakano and Mii (1992)
D. caryophyllus	L	SP	Nakano and Mii (1992)
D. chinensis	L	SP	Nakano and Mii (1992)
D. plumarius	L	SP	Nakano and Mii (1992)
Chenopodiaceae			
Spinacia oleracea	L	ShP	Goto and Miyazaki (1992)
Convolvulaceae			
Ipomoea batatas	L, St	ShP	Sihachakr and Ducreux (1987)
I. trifida	L	P	Suga et al. (1990)
I. triloba	L, St	ShP	Liu et al. (1991)
Cucurbitaceae			
Cucumus melo	L	E,ShP	Moreno et al. (1986)
C. sativus	Co	EP	Colign-Hooymans et al. (1988)
Ebenaceae			
Diospyros kaki	L	ShP	Tao et al. (1991)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
Euphorbiaceae			
Manihot esculenta	L	ShP	Shahin and Shepard (1980)
Fabaceae			
Crotalaria juncea	Co	E,ShP	Rao et al. (1982, 1985)
Dolichos biflorus	SC	E	Sinha and Das (1986)
Glycine argyrea	Co, L	ShP	Hammatt et al. (1989)
G. canescens	Co	P	Davey and Power (1988)
G. clandestina	Co	P	Davey and Power (1988)
	Co	ShP	Hammatt et al. (1987)
G. max	Co	ShP	Wei and Xu (1988)
G. soja	SC	E	Gamborg et al. (1983)
G. tabacina	SC	E	Gamborg et al. (1983)
Lotus corniculatus	Co, R	ShP	Ahuja et al. (1983a)
L. pedunculatus	Co	ShP	Pupilli et al. (1990)
L. tenuis	R	ShP	Piccirilli et al. (1988)
Lupinus mutabilis × *hartwegii*	L	Sh	Schafer-Menuhr (1989)
Medicago arborea	L	ShP	Arcioni et al. (1985a)
	L,R	ShP	Mariotti et al. (1984)
M. coerulea	L, SC	EP	Arcioni et al. (1982)
M. difalcata	Co	EP	Gilmour et al. (1987)
M. falcata	Co	EP	Gilmour et al. (1987)
M. glutinosa	L, SC	EP	Arcioni et al. (1982)
	Co	EP	Gilmour et al. (1987)
M. hemicycla	Co	EP	Gilmour et al. (1987)
M. sativa	L	EP	Johnson et al. (1981),
	L	P	Kao and Michayluk (1980)
	Co, R	EP	Lu et al. (1982b)
	C, SC	EP	Mezentsev (1981)
	L	E,ShP	Dos Santos et al. (1980)
	R	EP	Xu et al. (1982a)
Onobrychis viciifolia	L	ShP	Ahuja et al. (1983b)
Phaseolus angularis	L	E, ShP	Ge et al. (1989)
Pisum sativum	L, Co	Sh	Puonti-Kaerlas and Eriksson (1988)
Psophocarpus tetragonolobus	C	ShP	Wilson et al. (1985)
Stylosanthes macrocephala	Co	ShP	Vieira et al. (1990)
S. scabra	Co	ShP	Vieira et al. (1990)
Trifolium hybridum	L, R	ShP	Webb et al. (1984, 1986)
T. lupinaster	SC	ShP	Zhao et al. (1991)
T. pratense	Co, L	EP	Davey and Power (1988)

T. repens	L	ShP	Ahuja et al. (1983b)
	SC	ShP	Gresshoff (1980)
T. rubens	L, SC	EP	Grosser and Collins (1984)
Trigonella corniculata	L	EP	Lu et al. (1982a)
T. foennum-graecum	L	Sh	Shekhawat and Galston (1983a)
Vicia narbonensis	Sh	EP	Tegender et al. (1991)
Vigna aconitifolia	L	EP	Shekhawat and Galston (1983b)
V. mungo	L	E	Sinha et al. (1983)
V. sinensis	L	ES	Davey et al. (1974)
Gentianaceae			
Eustoma grandiflorum	L	ShP	Kunitake et al. (1990)
Geraniaceae			
Pelargonium × *domesticum*	L	ShP	Dunbar and Stephens (1991)
P. crispum	C	ShP	Miyazaki et al. (1992)
P. odoratissimum	C	ShP	Miyazaki et al. (1992)
Labiatae			
Pogostemon cablin	SC	SP	Sakurai and Kawachi (1990)
Linaceae			
Linum usitatissimum	Co, R	ShP	Barakat and Cocking (1983)
L. catharticum	Sh	ShP	Binding et al. (1992)
Magnoliaceae			
Liriodendron sulipifera	ES	EP	Merkle and Sommer (1987)
Malvaceae			
Gossypium barbadense	Co, R	E, ShP	Elishihy and Evans (1986)
G. hirsutum	ES	EP	Chen et al. (1989), She et al. (1989)
Myrtaceae			
Eucalyptus sp.	Sh	SP	Ito et al. (1990)
Oxalidaceae			
Oxalis glaucifolia	C	SP	Ochatt et al. (1989)
Passifloraceae			
Passiflora edulis	L	ShP	Manders et al. (1991)
Platanaceae			
Platanus orientalis	L	ShP	Wei et al. (1991)
Plumbaginaceae			
Limonium perezii	SC	ShP	Kunitake and Mii (1990a)
Polygonaceae			
Fagopyrum esculentum	Co	E, ShP	Adachi et al. (1989)
Primulaceae			
Cyclamen persicum	EC	EP	Otani et al. (1989)
Primula malacoides	SC	ShP	Mii et al. (1990)
Rauunculaceae			
Nigella arvensis	L	ShP	Binding et al. (1980, 1981)
N. sativa	Sc	ShP	Jha and Roy (1982)
Ranunculus sceleratus	L	EP	Dorion and Bigot (1985)
Rosaceae			
Fragaria ananassa	L	ShP	Binding et al. (1982)
Malus × *domestica*	C, SC	E	Kouider et al. (1984)
Prunus avium	L	SP	Ochatt (1991b)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
P. avium × *P. pseudocerasus*	L, SC	R, P	Davey and Power (1988)
P. cerasifera	L	ShP	Ochatt (1992)
P. cerasus	L, SC	R, P	Davey and Power (1988)
	L	R, P	Ochatt and Power (1988)
P. spinosa	L	ShP	Ochatt (1992)
Pyrus communis	L, SC	R, P	Davey and Power (1988)
	L	ShP	Ochatt and Caso (1986)
Rosa persica × *xanthina*	ES	EP	Matthews et al. (1991)
R. rugosa	EC	E	Kunitake and Mii (1990b)
Rubiaceae			
Coffea arabica	EC	EP	Yasuda et al. (1986)
	ES	EP	Acuna and de Pena (1991)
C. canephora	E	EP	Schopke et al. (1987, 1988)
Rutaceae			
Citrus aurantium	C	EP	Vardi and Spiegel-Roy (1982)
C. limon	C	EP	Vardi and Spiegel-Roy (1982)
C. madurensis	EC	EP	Ling et al. (1989)
C. paradisi	C	EP	Vardi and Spiegel-Roy (1982)
C. reticulata	C	EP	Vardi and Spiegel-Roy (1982)
C. sinensis	C	EP	Kobayashi et al. (1985),
	C	EP	Vardi and Spiegel-Roy (1982)
C. unshiu	EC	EP	Ling et al. (1990), Kunitake et al. (1991a,b)
Salicaceae			
Populus alba	L	ShP	Sasamoto and Hosoi (1990)
P. alba × *P. glandulosa*	L	ShP	Park and Son (1988)
P. alba × *P. grandidentata*	L	ShP	Russell and McCown (1986, 1988)
P. glandulosa	L	ShP	Park et al. (1990)
P. nigra	C	ShP	Lee et al. (1987)
P. nigra × *P. maximowiczii*	L	ShP	Park and Son (1989, 1992)
P. nigra × *P. trichocarpa*	L	ShP	Russell and McCown (1988)
P. sieboldii	L	ShP	Sasamoto and Hosoi (1990)
Santalaceae			
Santalum album	C	EP	Bapat et al. (1985)
	SC	EP	Rao and Ozias-Akins (1985)
Scrophulariaceae			
Antirrhinum majus	L	E	Poirier-Hamon et al. (1974)
Digitalis lanata	L	ShP	Li (1981)

D. obscura	L	ShP	Brisa and Segura (1987)
Paulownia fortunei	L	ShP	Wei et al. (1991)
Simarubaceae			
Ailanthus altissima	C	ShP	Park and Lee (1990)
Solanaceae			
Atropa belladonna	SC	EP	Gosch et al. (1975)
Capsicum annuum	L	ShP	Saxena et al. (1981b)
Cyphomandra betacea	Sh	ShP	Binding et al. (1992)
Datura innoxia	L	ShP	Schieder (1975, 1977)
D. metel	L	ShP	Schieder (1977)
D. meteloides	L	ShP	Schieder (1977)
Duboisia myoporoides	SC	ShP	Kitamura et al. (1989)
Hyoscyamus muticus	L,SC	ShP	Lorz et al. (1979)
	L	SP	Wernicke and Thomas (1980) Wernicke et al. (1980)
Lycopersicon chilense	SC	ShP	Hassanpour-Estamhbanati and Demarly (1986)
L. esculentum	Co	ShP	Koblitz and Koblitz (1982ab, 1983)
	C	SP	Morgan and Cocking (1982)
L. hirsutum	L	SP	Montagno et al. (1991)
L. pennellii	L, SC	ShP	Tan et al. (1987)
L. peruvianum	L	ShP	Muhlbach (1980)
	L	ShP	Zapata and Sink (1981), Zapata et al. (1977)
L. pimpinellifolium	Co	SP	Imanishi and Suto (1987)
Nicotiana acuminata	L	ShP	Bourgin et al. (1979)
N. alata	L	SP	Bourgin and Missonier (1978), Bourgin et al. (1979),
	L	ShP	Passiatore and Sink (1981)
N. debney	L	ShP	Piven (1981),
	L	SP	Scowcroft and Larkin (1980)
	L	ShP	Shakurov (1982)
N. glauca	L	ShP	Bourgin et al. (1979)
N. glutinosa	L	EP	Liu and Xu (1988)
N. langsdorfii	L	ShP	Bourgin et al. (1979),
	L	ShP	Evans (1979)
N. longiflora	L	ShP	Bourgin et al. (1979)
N. otophora	L	ShP	Banks and Evans (1976),
	L	ShP	Bourgin et al. (1979)
N. plumbaginifolia	L	ShP	Bourgin et al. (1979)
	L	ShP	Gill et al. (1978)
N. repanda	L	ShP	Evans (1979)
N. rustica	L	ShP	Gill et al. (1979)
N. sylvestris	L	ShP	Banks and Evans (1976)
	L	ShP	Bourgin et al. (1976, 1979),
	L	ShP	Nagy and Maliga (1976)
N. tabacum	L	ShP	Nagata and Takebe (1971), Takebe et al. (1971)
Petunia alpicola	C, SC	ShP	Ford-Logan and Sink (1988)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
P. axillaris	L	ShP	Power et al. (1976)
P. hybrida	L	ShP	Durand et al. (1973) Frearson et al. (1973)
P. inflata	L	ShP	Power et al. (1976)
P. parodi	L	ShP	Hayward and Power (1975)
P. parviflora	L	ShP	Sink and Power (1977)
P. violaceae	L	ShP	Power et al. (1976)
Salpiglossis sinuata	C	ShP	Boyes and Sink (1981), Boyes et al. (1980)
Solanum dulcamara	L	ShP	Binding and Mordhorst (1984), Binding and Nehls (1977), Binding et al. (1980, 1981)
S. etubersoum	L	ShP	Barsby and Shepard (1983)
S. melongena	L	ShP	Bhatt and Fassuliotis (1981),
	SC	ShP	Gleddie et al. (1982),
S. tuberosum	L	ShP	Binding et al. (1978), Bokdlmann and Roest (1983)
Sterculiaceae			
Theobroma cacao	SC	E	Kanchanapoom and Kanchanapoom (1991)
Tiliaceae			
Corchorus capsularis	Co, L	E	Saha and Sen (1992)
Ulmaceae			
Ulmus campestris	L	P	Dorion et al. (1991)
U. × *'Pioneer'*	C	ShP	Sticklen et al. (1986)

[a]Based on Roest and Gilissen (1989, 1993); for additional examples references are given in this book. C, callus, Co, cotyledon/hypocotyl; E, somatic embryos; EC, embryogenic callus; EP, plants regenerated via somatic embryogenesis; ES, embryogenic suspension; L, leaf; P, plantlet; R, root; SC, suspension culture; Sh, shoot; SP, plants regenerated via shoot bud differentiation; St, stem.

(1988) observed intervarietal differences for regeneration from protoplast-derived calli of indica rice. Of the 65 genotypes belonging to *Brassica oleracea*, *B. campestris*, *B. napus*, *B. juncea* and *Raphanus sativus* only 4 genotypes of *B. oleracea* and *B. napus* exhibited high plating efficiency (>35%), and regenerated shoots at variable frequencies. Other genotypes either failed to divide or divided with very low frequencies (<6%) and showed very poor regeneration if any (Jourdan and Earle, 1989). Genotypic variation also occurs for the pattern of regeneration (see Section 6.3.2).

12.4. CONCLUDING REMARKS

During the last 15 years considerable progress has been made in isolation and culture of protoplasts. With the available enzymes and protocols it is possible to isolate protoplasts from virtually any tissue of in vivo or in vitro growing plant materials. The progress during this period is particularly outstanding with regard to the number of species for which plant regeneration has been achieved from isolated protoplasts. This has been possible due to selection of right source tissue and plant genotype. A noteworthy achievement in this area has been isolation of viable protoplasts from egg and sperm cells of maize and their manipulation (see Section 10.4).

APPENDIX 12.I: PROTOCOLS FOR ISOLATION AND CULTURE OF PROTOPLASTS OF FOUR SPECIES

12.I.1. *Nicotiana tabacum*

(a) Select fully expanded leaves from 7–8-week-old plants growing in a glasshouse.
(b) Surface-sterilize the leaves by first immersing in 70% ethanol for 30 s followed by rinsing in 0.4–0.5% sodium hypochlorite solution for about 30 min.
(c) Wash the leaves thoroughly with sterile distilled water to remove every trace of hypochlorite.
(d) Peel the lower epidermis with fine forceps and cut out the peeled areas with a fine scalpel.
(e) Place the peeled leaf pieces on a thin layer of 600 mmol l^{-1} mannitol-CPW solution[3] in such a way that the peeled surface is in contact with the solution.
(f) After about 30 min replace the mannitol/CPW solution by filter-sterilized enzyme solution containing 4% cellulase SS, 0.4% macerozyme SS, 600 mmol l^{-1} mannitol and CPW salts.
(g) Seal the petri plate with parafilm and incubate it in the dark at 24–26°C for 16–18 h.
(h) Gently squeeze the leaf pieces with a Pasteur pipette to liberate the protoplasts.

[3] Cell-protoplast washing medium (CPW) contains (mg l^{-1}); KH_2PO_4 (27.2), KNO_3 (101), $CaCl_2 \cdot 2H_2O$ (1480), $MgSO_4 \cdot 7H_2O$ (246), KI (0.16), $CuSO_4 \cdot 5H_2O$ (0.025), pH 5.8 (Cocking and Peberdy, 1974).

(i) Remove the large debris by filtering through a 60–80 μm nylon mesh.
(j) Transfer the filtrate to a screw-cap centrifuge tube and spin at 100 × *g* for 3 min.
(k) Remove the supernatant and transfer the sediment, on the top of 860 mmol l^{-1} sucrose solution (prepared in CPW) in a screw-cap centrifuge tube and spin it at 100 × *g* for 10 min.
(l) Collect the green protoplast band from the top of the sucrose pad and transfer it to another centrifuge tube.
(m) Add the protoplast culture medium (e.g. NT medium; for composition see Table 3.1) to suspend the protoplasts and centrifuge at 100 × *g* for 3 min. Repeat such washings at least three times.
(n) After the final washing add enough culture medium to achieve a protoplast density of 0.5×10^5 to 1×10^5 ml^{-1}.
(o) Plate the protoplasts as small (100–150 μl) droplets or a thin layer in petri plates.

12.I.2. *Arabidopsis thaliana* genotype C24 (after Damm and Willmitzer 1988, 1991)

(a) Take 1–2 g of leaf material from 3 to 4-week-old aseptically growing plants and place them in a 94 × 16 mm petri plate so that the lower side of the leaves is towards the bottom of the plate, and wet them with 4–7 ml of 0.5 M mannitol solution, in dark, at 25°C.
(b) Cut the leaves with a razor blade so that the leaf is cut once.
(c) Transfer the leaf material into two plates, each containing 10 ml of 0.5 M mannitol and plasmolyse them for 1–2 h in the dark at room temperature.
(d) Remove the mannitol solution and replace it with 12 ml of enzyme solution containing 1% cellulase 'Onozuka' R-10, 0.25% Macerozyme R-10, 8 mmol l^{-1} $CaCl_2$, 0.4 M mannitol (pH 5.5). Incubate the plates in dark at 25°C.
(e) After 16–20 h agitate the mixture and wait for another 30 min to complete the digestion.
(f) Separate the protoplasts from the undigested tissue by consecutive filtration of the mixture through a 125 μm stainless steel sieve on top of a 63 μm sieve. Wash the petri plate with 6 ml of 0.2 M $CaCl_2$ in order to recover the remaining protoplasts and add this solution to the filtrate through the sieve.
(g) Distribute the filtrate into four 12 ml centrifuge tubes and spin for 5 min at 60 × *g* in a swinging bucket rotor.

(h) Carefully remove the supernatant with a pipette and resuspend the pellet in a solution containing 3 ml of 0.5 M mannitol and 6 ml of 0.2 M $CaCl_2$ and centrifuge for 5 min at 40 × *g*.
(i) Repeat the washing of protoplasts as in step (h).
(j) Suspend the protoplasts of each centrifuge tube in a solution composed of 2 vols. of 0.5 M mannitol and 1 vol. of 0.2 M $CaCl_2$ and recentrifuge at 40 × *g*.
(k) Finally, suspend the protoplasts in 0.5 M mannitol at a density of 4–6 × 10^5 ml^{-1} and put on ice in dark for at least 30 min to stabilize the protoplasts.
(l) Embed the protoplasts in sodium alginate as follows (all solutions used are to be cooled in ice): mix equal volumes of 0.4 M mannitol solution containing 2.8% sodium alginate and the protoplast suspension. Add 1 ml of this mixture dropwise to 3 ml of solution 1 (50 mmol l^{-1} $CaCl_2$ in 0.4 M mannitol) in small (60–15 mm) petri dishes. Due to the presence of $CaCl_2$ Ca-alginate beads will be formed. After 1–2 h at room temperature replace the solution 1 by 3 ml of solution 2 (10 mM $CaCl_2$ in 0.4 M mannitol).
(m) After keeping the embedded protoplasts for 1–2 days at 4°C in the dark replace solution 2 by 3 ml of B_5 medium containing 0.4 M glucose, 1 mg l^{-1} 2,4-D and 0.15 or 0.5 mg l^{-1} BAP and incubate the plates at 26°C in the dark for 3 weeks. Renew the medium every 10 days.
(n) After 3 weeks add fresh medium and transfer the plants to light (700 lx) at 26°C. Renew medium every 2 weeks.
(o) After 5–7 weeks free the protoplast derived colonies by incubating the beads in a solution containing 0.3 M mannitol and 20 mM sodium citrate.
(p) Transfer the larger colonies to MS medium containing 2% sucrose, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA, and incubate in the light (16/8 h photoperiod) at 25°C, for shoot differentiation.
(q) After 2–5 weeks transfer the shoots to shoot elongation medium (MS + 0.1 mg l^{-1} NAA, 1 mg l^{-1} BAP, 0.1 mg l^{-1} GA_3) and incubate at 21°C under photoperiod as in step (p).
(r) After another 8–10 weeks, root the shoots on a medium containing MS salts and vitamins at half strength, 1% sucrose and 1 mg l^{-1} IBA.

12.I.3. *Brassica napus* cv *isuzu* (after Chuong et al., 1985)

(a) Raise aseptic seedlings on 0.8% agar supplemented with 0.2% sucrose, in the dark, at 25°C.

(b) Excise 150–200 hypocotyl hooks (2–3 cm long) from 2-day-old seedlings and plasmolyse them for 1 h in CPW solution (for composition see Appendix 12.I.1) supplemented with (in mg l^{-1}) ampicillin (400), gentamycin (10), tetracyclin (10) and 13% mannitol ('CPW 13 M'), with pH set at 5.7.

(c) Transfer the hypocotyl pieces to the enzyme solution containing 2% Rhozyme HP-150 (Genecor, N.Y., USA), 4% meicelase (Meiji Seika Co., Tokyo, Japan) and 0.3% macerozyme R-10 (Yakult Pharmaceutical Co., Nishinomya, Japan) in CPW 13 M and incubate at 25°C in the dark on a shaker at 60 rev. min^{-1}.

(d) After 12 h of enzyme treatment gently agitate the mixture by taking up into and expelling from a pipette several times to enhance the release of the protoplasts.

(e) Filter the enzyme mixture through two layers a nylon mesh (60 μm pore size on top of 44 μm).

(f) Transfer the filtrate to centrifuge tubes and spin at 100 × g for 3 min.

(g) Suspend the pellet in CPW 13 M and spin again. Repeat this washing process three times.

(i) Finally, suspend the protoplast pellet, at a density of 2×10^5 protoplasts ml l^{-1}, in a modified Lichters medium[4], supplemented with 13% w/v sucrose, 5 g l^{-1} Ficoll 400 (Pharmacia Fine Chemicals, Sweden), 0.5 mg l^{-1} BAP, 1 mg l^{-1} NAA and 0.5 mg l^{-1} 2,4-D, with pH set at 5.7 and plate them in petri plates (2.5 ml of protoplast suspension per 15 × 60 mm petri plate. Incubate the plates at 25°C in dark.

(j) After 4–6 weeks transfer the floating microcalli to MS medium containing 200 mg l^{-1} casein hydrolysate, 5 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 0.6% agarose, with pH set at 5.7.

(k) After 3–4 weeks shoot buds differentiate.

12.I.4. *Oryza sativa* (*Indica* cvs *Nipponbare* and *Iwaimochi*) (after Kyozuka et al., 1987)

(a) Surface sterilize the mature seeds and plant them on MS medium supplemented with 2 mg l^{-1} 2,4-D.

---

[4] Composition of Lichter's medium (mg l^{-1}): $Ca(NO_3)_2 \cdot 4H_2O$ (500), KNO_3 (125), $MgSO_4 \cdot 7H_2O$ (125), KH_2PO_4 (125), $MnSO_4 \cdot 4H_2O$ (25), H_3BO_3 (10), $ZnSO_4 \cdot 4H_2O$ (10), $Na_2MoO_4 \cdot 2H_2O$ (0.25), $CuSO_4 \cdot 5H_2O$ (0.025), $CoCl_2 \cdot 6H_2O$ (0.025), EDTA (0.037), $FeSO_4 \cdot 7H_2O$ (0.028), glycine (2), myo-inositol (100), nicotinic acid (5), pyridoxin·HCl (0.5), thiamin·HCl (0.5), folic acid (0.5), biotin (0.05), glutathione (30), L-glutamine (800) and L-serine (100).

(b) After 3–4 weeks transfer the scutellar callus to 125 ml flask containing 25 ml of liquid medium containing inorganic salts of R-2 medium[5], 5.6 mg l^{-1} $FeSO_4$, 7.5 mg l^{-1} Na_2EDTA, vitamins of MS, 1 mg l^{-1} 2,4-D and 3% sucrose, and place the flask on a shaker at low speed (50 rev. min^{-1}). Suspension can be maintained by weekly subcultures.

(c) Filter the cell suspension through a sieve of 500 μm pore size into a pre-weighed 10 cm plate. Remove the medium with a sterile pipette, leaving the cells in the plate and add (10 ml^{-1} g^{-1} fresh weight of cells), the enzyme mixture consisting of 4% cellulase RS (Kinki Yakult, Japan), 1% macerozyme R-10 (Kinki Yakult, Japan) and 0.4 M mannitol. Seal the plate with parafilm or nescofilm and incubate it in the dark at 30°C without shaking.

(d) After 3–4 h filter the enzyme mixture through a 20 μm nylon mesh and add four times the volume of KMC solution, consisting of equal volumes of 0.35 M KCl, 0.245 M $MgCl_2$ and 0.254 M $CaCl_2$, and pH set at 6.

(e) Centrifuge for 10 min at 800 rev. min^{-1}.

(f) Wash the pelleted protoplasts twice in KMC solution by centrifugation.

(g) Mix 1 ml of the protoplast suspension (1×10^6 protoplasts ml^{-1}) in culture medium (basal medium used for suspension cultures containing 2 mg l^{-1} 2,4-D and 0.4 M sucrose (pH 5.0) with an equal volume of the molten agarose medium (2.5% Sea Plaque agarose) in 6 cm petri plate.

(h) Cut the solidified agarose, containing the protoplasts, into 8 × 8 mm blocks and transfer them to 6 cm plate containing 5 ml of protoplast culture medium.

(i) Add rice Oc nurse cells (100 mg/plate) to the liquid part of the culture (mixed nurse method).

(j) After 10 days transfer the agarose blocks to new plates with nurse cells-free medium. Completely remove the nurse cells from agarose blocks by washing with the culture medium.

(k) After 4 weeks transfer the agarose blocks containing visible colonies to soft agarose medium containing N_6 basal medium (for composition see Table 7.2) supplemented with 2 mg l^{-1} 2,4-D, 6% sucrose and 0.25% agarose, and culture under light (3000 lx).

---

[5] Composition of R-2 medium (mg l^{-1}): $NaH_2PO_4.2H_2O$ (307.78), KNO_3 (4040), $(NH_4)_2SO_4$ (330), $MgSO_4.7H_2O$ (256.32), $CaCl_2$ (110.9), Fe-EDTA (2.5), $MnSO_4.H_2O$ (0.5), $ZnSO_4.7H_2O$ (0.5), H_3BO_3 (0.5), $CuSO_4$ (0.05), $Na_2MoO_4.2H_2O$ (0.05), $CoCl_2.6H_2O$ (0.05), thiamine-HCl (1), sucrose (2%), 2,4-D (2), pH 6.

(l) After 2–3 weeks transfer individual colonies (Ca 1 mm in diameter) to the medium as in step (k) but with higher agarose concentration (0.5%).

(m) When the colonies attain a size of about 2 mm transfer them to the N_6 basal medium containing 6% sucrose, 1% agarose and 2 mg l^{-1} kinetin or 5 mg l^{-1} BAP.

(n) After 3–8 weeks transfer the regenerated shoots to hormone-free N_6 medium for rooting.