1 Culture Systems and Selection Procedures

Hamish A. Collin and Philip J. Dix

1.1 Introduction

Plant tissue culture provides a powerful technique to assist the plant breeder in improving the propagation and performance of agricultural, horticultural and forest species, to provide the industrialist with a potential source of pharmaceuticals, safe colours and flavours, and to supply the biochemist with standardised plant material for the analysis of plant metabolism. The actual contribution that tissue culture has made to these fields of endeavour is variable, and is a principal theme of the review chapters of this book, but the ability to distinguish specific phenotypes at the cell level is a recurrent feature of the diverse fundamental and applied investigations in this area. For example, *in vitro* selection may lead to the production of plants showing resistance (or tolerance) to specific environmental stresses, such as low temperature, salinity, drought, herbicides, toxic metals, and pathogens, the production of resistant cell lines for analysis of the resistant mechanism at the cellular level and the selection of cell lines for high yields of secondary products such as alkaloids, steroids, terpenes, colourings and flavourings. In addition, contemporary approaches to genetic recombination, such as cell fusion and transformation, are dependent on our ability to identify the desired recombinants.

In any review on plant cell line selection, whether it is a single chapter (eg, Flick, 1983; Maliga, 1984; Dix, 1986) or a complete monograph, as in this case, there are two fundamental pieces of groundwork which need to be laid. These relate to the range of tissue culture procedures which may be appropriate to any particular selection objective, and the choice of approach to selection most suitable for realising that objective. A consideration of these basic aspects of *in vitro* selection is the purpose of this chapter. Our intention is that a brief review of the types of tissue culture, and selection strategies, will lay the foundation for a fuller appreciation of the topics covered by the remainder of the text. Our aim is not to provide a detailed review of tissue culture methods. Clearly that is beyond the scope of the present volume, particularly since the species specificity of response to culture *in vitro* would necessitate a crop by crop approach of the kind adopted in an earlier multi-volume treatise (Sharp *et al.*, 1984, Ammirato *et al.*, 1984). There are a number of other volumes devoted to tissue culture (eg, Vasil, 1984; Dixon, 1985; Walker and Pollard, 1990) and many of these lay an emphasis on detailed experimental protocols. It is, however, our hope that the material included here will be of value to "new" tissue culture workers, including those attracted from other fields by the exciting prospects now becoming apparent in plant biotechnology, for example the combination of tissue culture and molecular biological techniques for the genetic engineering of crop plants (see Chapter 13).

In all the past and present studies using *in vitro* selection, the approach was based on the assumption that plant cell cultures did not consist of a large number of uniform cells, but were a population of cells showing a range of genotypes. Cell cultures can be regarded, therefore, as the same as any animal or plant population. There is variation in individual genotype, phenotype and age. The presence of variation in tissue culture cells is shown more clearly when the cells are regenerated into plants. The regenerant plants demonstrate a wide variation in colour, leaf shape, shoot morphology, size and resistance to disease, herbicides, etc. If the plants are allowed to flower, set seed and variation in the progeny examined, some of the changes shown by the regenerant plants will be seen in the progeny, ie, the variation is stable through a seed generation. This variation, known as somaclonal variation (Larkin and Scowcroft, 1981) is thought to arise partly from somatic mutations present in the original explant but is increased in culture from somatic mutations generated during callus initiation and subculture.

It can be further increased by the use of mutagenic agents (Chapter 2) and the kind of genomic mixing achieved through protoplast fusion (Chapters 11 and 12) and transformation (Chapter 13). Our interest here is in selecting out useful forms of this variation, in culture, without the need for screening among regenerated plants or their progeny. As a prelude to considering the tissue culture systems it is worth outlining the different approaches which may be adopted to achieve this selection. The feasibility of these can then be related to the characteristics of the different cultures.

1.2 Selection Strategies

1.2.1 Resistance Selection

This is certainly the most widely practised approach to selection *in vitro*. It can be used whenever conditions can be defined under which the required phenotype has a selective advantage over wild type cells. The mass culture is simply incubated under these conditions (eg, toxic levels of drugs) or after being subjected to them (eg, chilling). Only resistant cells will grow, and hence resistant lines can be recovered. The approach, in one form or another, is widely used to obtain lines resistant to disease (Chapter 5), herbicides (Chapter 6), environmental stresses (Chapter 7), and also to obtain useful genetic markers (Chapter 9), and to select nuclear (Chapter 11) and cytoplasmic (Chapter 12) hybrids, and transformed cells (Chapter 13). In some of these latter cases "resistance" may take the form of restoration of an ability to grow under certain conditions, such as complementation between auxotrophic and/or nitrate reductase deficient lines in somatic hybrids (Chapter 11).

1.2.2 Visual Selection

This is applied when the required phenotype has no selective advantage over wild type cells, yet can readily be identified visually, permitting rapid screening of, for example, colonies growing on petri plates, or cells in suspensions. Most commonly it applies to accumulation of a pigment, such as anthocyanin (eg. Dougall *et al*, 1980), or loss of a pigment, such as chlorophyll (eg. Svab and Maliga, 1986). Another form of visual selection is visual identification and isolation of heterokaryons after protoplast fusion (Menczel *et al*, 1978) (Chapters 11 and 12), and cell sorting on the basis of optical characteristics (Chapter 3) may be considered as the automated version. Some selection protocols, such as those for obtaining antibiotic resistant cell lines (Maliga *et al*, 1973, Cseplö and Maliga, 1982) combine features of resistance and visual selection.

1.2.3 Counterselection

For auxotrophs, and some other conditional lethal mutants there is no straightforward approach to selection. Conditions cannot be devised which favour the growth of mutant compared to wild type cells. Counterselection is an approach which has been developed to circumvent this problem. It is based on the fact that certain drugs will kill only dividing cells, so that others, division of which is inhibited, for example by a nutritional deficiency, will survive. The population is enriched for such cells by their subsequent transfer to permissive medium on which they can grow. The procedure was devised for animal cells (Puck and Kao, 1967) where the counterselective agent used was the thymidine analogue bromodeoxyuridine. Attempts to use a similar approach with plant cells have had a chequered history and are reviewed in Chapter 10 (see also Dix, 1986).

1.2.4 Total Selection

Here, we refer to the systematic screening of large numbers of cell colonies for non-selectable characters. Generally part of each colony is removed for testing. Typical screening procedures are growth tests on non-permissive medium, to identify auxotrophs (see Chapter 10) or chemical or immunological tests, to identify lines, for example, producing secondary metabolites (Chapter 8).

1.2.5 Micromanipulation

A fundamentally different approach is now available for the genetic manipulation of plant cells by, for example, protoplast fusion or transformation. Instead of working with mass populations of cells, and selecting out the desired products, selection is manifested, as the

experimenter's choice of individual target cells. These can be manipulated and cultured independently without recourse to external selection pressures, or screening. A detailed consideration of the applications of this approach, as well as a description of the instrumentation and procedures, is the subject of Chapter 4.

1.3 *In vitro* Culture Systems

The nature of the tissue culture material used in the selection is important as it may determine the stability of the character and the regenerative capacity of the culture. In selecting for resistant mutants for a programme of crop improvement, for example, it is essential to be able to regenerate plants from resistant cells. The material available for selection may vary from simple single cells to complex differentiated tissues. The choice of species and tissue culture material is often determined by availability of the plant species, and the routine nature of the protocol for producing a particular tissue culture. In evaluating the use of different types of tissue culture for selection we shall, in keeping with the continuing development of this area, illustrate their use with reference to the recent literature.

1.3.1 Callus Cultures

Callus consists of a mass of tissue, with a low level of organisation, obtained by transferring pieces of plant organs (eg, internode, leaf or root) to a suitable nutrient medium, frequently containing appropriate combinations of plant growth regulators. Commonly, callus can be maintained indefinitely by regular transfer to fresh culture medium and ideally plants can be regenerated from it, through adventitious shoot initiation or somatic embryogenesis. Poor capacity for regeneration is, however, frequently a stumbling block in the use of callus for genetic manipulations. Callus is an immobilised system, insofar as it is usually grown on a solid base, for example medium solidified with agar. Individual calli can therefore, if necessary, be identified, and their growth and properties be monitored over substantial periods of time.

Callus cultures are extremely commonly used for selection *in vitro*. Resistance selection, for a number of traits, is widely performed in callus cultures, and they are also appropriate for visual selection and total selection. Under some circumstances, selection of somatic (Chapter 11) or cytoplasmic (Chapter 12) hybrids as well as transformed cells (Chapter 13) might best be performed at the level of callus, although the initial genetic manipulations involve the use of protoplasts.

Resistance selection in callus cultures is particularly straightforward to perform. Selected cells surrounded by a large number of dead cells in the callus piece grow out as an easily identifiable lump on the side of the callus. The new growth can be excised and sub cultured. Where the normal growing callus is green, the growing areas can be more easily identified (Jordan and McHughen, 1987).

A criticism of the use of callus in a selection procedure is the potential risk of producing chimaeras. One way of reducing this risk is to use very small pieces of callus. For example, Ibrahim (personal communication) in selecting for salt tolerance in *Coleus blumei* incubated 50 small even sized pieces of callus (15 mg) on selection medium in a 9 cm diameter petri dish (Fig. 1-1). In screening for salt tolerance in other species callus pieces 100 mg, *Beta vulgaris* (Pua and Thorpe, 1986), 20 mg *Brassica napus* (Chandler and
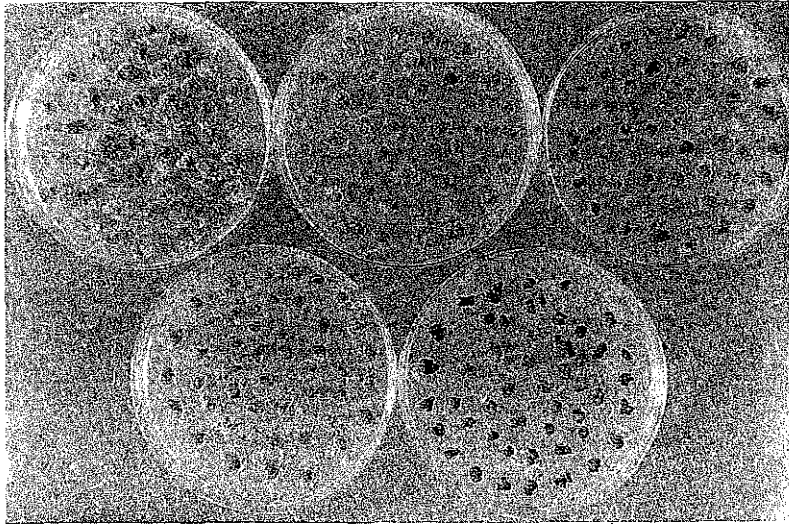

Fig. 1-1. Early stages in selection for improved salt tolerance in callus cultures of *Coleus blumei* Small callus pieces (15 mg) are placed on medium containing a range of NaCl concentrations: Top (left-right) 0, 30 and 60, and Bottom (left-right) 90 and 120 mM (courtesy of K. Ibrahim).

Thorpe, 1987) and 150 mg, rice (Kavi Kishor, 1988) were used. In selection for 5-methyl-tryptophan resistant rice (Wakasha and Widholm, 1987) 10-20 mg callus pieces and for chlorosulfuron resistance in *Linum usitatissumin* (flax) (Jordan and McHughen, 1987) pieces of callus 1-2 mm diameter were screened.

The selection procedure has been based on either a direct or a stepwise approach. Most of the selections involved the direct approach, where the callus was exposed to one concentration of selective agent, then the surviving cells isolated and bulked. This procedure was adopted for the selection of resistance of salt (McHughen and Swartz, 1984; McHughen, 1987; Chandler and Thorpe, 1987; Kavi Kishor, 1988), amino acid analogues (Wakasa and Widholm, 1987; Schaeffer and Sharpe, 1987), herbicides (Jordan and McHughen, 1987) and heavy metals (Khadeeva *et al.*, 1985), and selection for lactose utilisation (Elavummoottil *et al.*, 1988).

The alternative is to use the less favoured but equally effective stepwise approach where the surviving callus is sequentially transferred to increasing levels of the selective agent. Ibrahim (personal communication) transferred callus of *Coleus blumei* from 30 mM NaCl with a stepwise increase of 15 mM at each 3 week culture period to 150 mM NaCl. Callus of *Beta vulgaris* were transferred in a similar way to successively higher concentrations of Na_2SO_4 with a final concentration of 210 mM (Pua and Thorpe, 1986), and tobacco callus resistant to 2,4-dichlorophenoxy acetic acid was obtained in the same

way (Nakamura *et al.*, 1985). A combination of both approaches was employed by Chawla and Wenzel (1987) in selecting for resistance to *Helminthosporium sativum.* Two selection methods were employed. In the continuous method small pieces of callus (30 mg) were placed on medium containing purified toxin, then transferred to fresh toxic medium every three weeks for four subcultures. In the discontinuous method, the callus was placed on a non-toxic medium after the second subculture, then a further two subcultures on the toxic medium in order to retain the capacity for regeneration in selected callus.

Stability in callus cultures which have been selected for resistance was demonstrated by survival of the selected callus for an extended period in the selective medium, or on return to it after an extended period in a non selective medium or non selective conditions. Salt resistant cultures of *Citrus aurantinum,* for example, were maintained for one year in a salt containing medium before examination of the tolerance mechanism (Ben-Hayyim *et al.*, 1985). Similarly selected *Beta vulgaris* cultures were maintained on a high Na_2SO_4 medium for nine months before analysis of salt tolerance (Pua and Thorpe, 1986). In other studies of salt tolerance, selected rice cultures were first incubated on a salt free medium for 10 subcultures (Kavi Kishor, 1988), *Cicer arietinum* for six subcultures (Pandey and Ganapathy, 1985) and cultures of *Brassica napus* for thirteen subcultures (Chandler and Thorpe, 1987) before characterising the resistance mechanism. The resistance to salt was retained despite this period in non selective conditions. Cultures of the dihaploid potato however selected for resistance to 5-methyl-tryptophan showed some loss in resistance after five months in a non selective medium (Jacobsen *et al.*, 1985). No plant regeneration was described for any of these cultures.

Providing the callus is recently initiated it is however possible to regenerate selected resistant cultures and test for the presence of resistance in the regenerant plants and progeny. For example, salt tolerance in flax cultures was detected in regenerated plants and in the progeny (McHughen and Swartz, 1984; McHughen, 1987). Chlorsulfuron resistant flax cultures were also regenerated and the progeny tested (Jordan and McHughen, 1987). However, instead of using intact plants for the progeny test, resistance was demonstrated in callus cultures initiated from the progeny.

Callus tissue has also been used in selection for secondary metabolite production where it is possible to carry out a visual selection. Thus high anthocyanin producing cell colonies have been identified and subcultured in callus cultures of sweet potato, *Ipomoea batatas* (Nozue *et al.*, 1987) and *Bubleurum falcatum* (Hiraoka *et al.*, 1986).

These cell lines were surprisingly stable in the absence of selection. Thus Nozue *et al.*, (1987) found that selected lines of sweet potato cultures showed high anthocyanin levels after 35 subcultures, and *Bupleurum falcatum* maintained high productivity for three years after selection (Hiraoka *et al.*, 1986). Clearly the anthocyanin producing cells are not at a disadvantage in the cell population.

1.3.2 Cell Suspension Cultures

Cell suspension cultures are most commonly obtained by dispersing friable callus cultures in liquid culture medium, and agitating on a rotary shaker, although they can also be initiated directly from explants. Suspension cultures usually become more finely dispersed as the culture period is prolonged, but degree of dispersion, and growth rate, are dependent on both the species, and the culture conditions. Suspension cultures can be maintained

using a wide range of culture methods, ranging from shaker flasks to large scale fermenters.

A cell suspension which is well dispersed provides a suitable tissue culture for selecting for resistant mutants since the single cells and cells in the small cell aggregates, are uniformly exposed to the selective agent. As with callus, selection has involved direct inoculation of the cells into a medium containing a toxic concentration of the selective agent (eg, salt or herbicides), or gradual, stepwise increases. In the former case one or more colonies may develop from the limited number of resistant cells in the original population. A protracted period in culture is often necessary because growth from one, or a small number of cells, has an extended lag phase. Kishinami and Widholm, (1986) incubated cells of *Nicotiana plumbaginifolia* in liquid medium containing toxic levels of $CuSO_4$ (0.02 mM) and $ZnSO_4$ (2 mM). After two to three months pale yellow clumps, thought to be of single cell origin were noted.

A stepwise approach, in which the cell suspension is subcultured into medium containing increasing levels of selective agent is perhaps more widely used with cell suspension cultures. This allows both adaptation and selection to occur. Since the increases in selective agent are relatively small, in the initial stages there are always a number of cells in the inoculum which can tolerate the new concentration. There is therefore only a short lag phase and cells can be transferred quickly into each new concentration. The lag phase however will increase towards the ceiling concentration. The main areas of selection where the stepwise approach has been used are the selection for resistance to salt, herbicides and heavy metals. In a number of recent papers on salt tolerance in *Nicotiana tabacum*, Hasegawa and co-workers made use of a technique outlined earlier (Hasegawa *et al.*, 1980). Cell suspensions were subcultured in a stepwise manner from 10 g/L to 14, 20, 25 and finally 35 g/L (599 mM). Cells were maintained for 50 generations in each medium before transfer to the next concentration. This procedure allowed the selected cells to be bulked up and form a good inoculum for the next subculture. It is very important to do this as lack of growth during one subculture will reduce the size of the inoculum and extend the lag phase of the following subculture. If the inoculum is too low the cell suspension will not divide. Watad *et al.* (1983) used a similar stepwise approach to select for salt resistance in *Nicotiana tabacum*. Callus was trasnferred to agar medium containing a low salt concentration (85 mM) then surviving pieces of callus were transferred to liquid medium containing the same salt concentration, with 10 mM $CaCl_2$ added to maintain the integrity of the cell membrane. Cells were subcultured every four weeks until cell lines growing in 400 mM were achieved. More recently tolerance levels of 500 mM were obtained by the same method (Watad *et al.*, 1985).

In selecting for glyphosate tolerance in cultures of *Daucus carota*, carrot, Nafziger *et al.*, (1984) transferred cells initially to 0.25 mM glyphosate then into progressively higher concentrations until on a medium with 25 mM glyphosate. Similarly, glyphosate tolerant *Petunia hybrida* cells were established by successive subculture in a medium containing 0.5, 1.0, 2.5, 5.0, 7.0 and finally 10 mM glyphosate (Steinrucken *et al.*, 1986). The same approach was adopted by Huang *et al.*, (1987) to produce cadmium tolerant cells of *Lycopersicon esculentum*. The cadmium (as $CdCl_2$) in the culture medium was progressively elevated from 200 mM to 500, 1000, 1200, 1500, 2000, 3000, 4000 and 5000 mM cadmium, then subcultured at the highest concentration for six months before being described as adapted.

For most variant cell lines, obtained through selection in cell suspension cultures, plant regeneration has not been reported. This may be a feature of the loss of capacity to regenerate, frequently associated with long term cultures. A protracted culture period is

frequently needed to obtain a cell suspension suitable for selection, and then the selection process itself may also take a long time. Generally, the use of cell suspension cultures has not therefore been favoured for genetic manipulations. Difficulties in regeneration from cell suspension cultures are not however universal. For example, hydroxyproline resistant potato plants have been obtained after selection in such cultures (van Swaaij *et al.,* 1986).

In the absence of plant regeneration, stability of the selected trait has been assessed by examining whether it is still expressed after a long period of subculture in the absence of the selection pressure, an approach which cannot be regarded as providing evidence for a genetic, rather than epigenetic, basis for the change (Dix, 1986). Stability of the trait is also a factor which needs to be assessed where selected cells are used to examine the mechanisms of resistance. In selecting cultures of *Nicotiana tabacum* for salt resistance, Hasegawa *et al.* (1980) found that salt resistant lines were unstable and consequently in later work the analysis of the resistance mechanism was made on resistant cells that had been maintained without a break on a medium containing 10 g/L NaCl (Binzel *et al.,* 1985, 1987). However, some salt resistant lines of tobacco were stable and were maintained for 56 generations on a salt free medium before analysis (Watad *et al.,* 1985). Selection for resistance to heavy metals appear to be stable since cultures of *Lycopersicon peruvianum* were still resistant to cadmium after 30 generations (Bennetzen and Adams, 1984), and cultures of *Lycopersicon esculentum* after 12 months (Huang *et al.,* 1987), and *Nicotiana plumbaginifolia* was considered to be resistant to copper and zinc after 30 cell generations (Kishinami and Widholm, 1986). The same approach was adopted in examining herbicide tolerance in selected cells. Glyphosate resistant cultures of *Daucus carota* still showed resistance after 120 generations in nonselective medium (Nafziger *et al.,* 1984) and selected cells of *Petunia hybrida* after prolonged culture in a low glyphosate containing medium (Steinrucken *et al.,* 1986).

Investigations aimed at tapping the biosynthetic capacity of plant cells, by producing lines accumulating valuable secondary metabolites, clearly have no requirement for plant regeneration, although stability of the trait remains of paramount importance. In these cases selection is commonly performed in cell suspension cultures, especially since cell suspensions remain the most convenient system for large scale culture of plant cells (see Chapter 8).

1.3.3 Plated Cell Suspensions

A modification to the use of cell suspensions in *in vitro* selection has been to grow the cells first in liquid culture then transfer them to a petri dish onto or in a medium containing the selective agent. The purpose of the plating step is to expose the single cells, and small cell aggregates of the cell suspension, to the selective agent, and then to be able to identify and remove individual resistant colonies. One practical advantage of the plating technique is that the screening can be carried out on a large scale with minimum space requirements. The results can also be very easily visualised and photographed (Fig. 1-2).

The main problem is to ensure a good contact between the selection medium and the cells. This has been achieved in a number of ways. At its simplest the suspension is transferred to the surface of solidified agar containing the selective agent. This proved to be quite effective for the selection of zinc resistant cell lines of *Haplopappus gracilis* (Gilissen
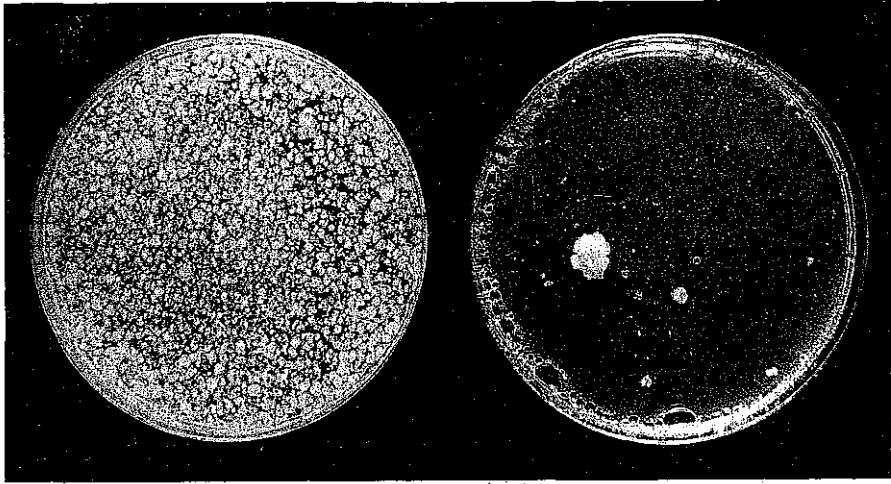
Fig. 1-2. Selection for resistance to hydroxyproline in plated cells of *Nicotiana plumbaginifolia.* Suspension cultures were mixed with molten agar medium (40°C) and poured into petri dishes. Medium contains 0 (left) or 10 mM (right) hydroxyproline. Dishes were photographed after 8 weeks.

and Van Staveren, 1986), and lines of dihaploid potato resistant to 5-methyl-tryptophan (Jacobson *et al.*, 1985) and to S-(2-aminoethyl)-cysteine (Jacobson, 1986). To improve the contact between the cells and selective agent, the cell suspensions have been transferred first to liquid medium containing a selective agent then plated on or in agar also containing a selective agent. Thus Flashman *et al.* (1985) selecting for vernolate tolerance in *Nicotiana tabacum* maintained the cell suspension in a vernolate containing medium for 24 hours before plating on agar. Similarly, Singer and McDaniel (1985) resuspended the cells in liquid medium containing 1 mM glyphosate before transferring the suspension to agar medium containing 1 mM glyphosate. After two months growing colonies were transferred to fresh medium. Alternatively, cell suspensions of *Daucus carota* were layered onto semisolid agar (0.6%) containing the selective agent, methotrexate (5-6 × 10^{-4}M), (Cella *et al.*, 1987). Surviving colonies with a diameter bigger than 2 mm where then transferred to a maintenance medium. A further sophistication was that used by Flashman (1985), selecting for herbicide tolerant cell lines of *Nicotiana tabacum.* Suspension culture cells were resuspended in a medium containing 0.8% low gelling temperature agarose containing a selective agent, a non volatile thiocarbamate to select lines showing cross tolerance to the commercial thiocarbamates. The resuspended cells were layered onto a medium containing the herbicide at the same concentration and solidified with ordinary agar (0.6%). After one month colonies were trasnferred to fresh medium.

The prolonged growing period of the plated cells does not seem to have presented particular problems. None of the reports for instance refer to the need to overcome the inhibitory effects of low density on the growth of the few surviving resistant cells. Drying out of the medium in these long term cultures may be more of a problem. This can be avoided by adopting the procedure of Ibrahim (personal communication) in selecting for salt tolerance in *Coleus blumei.* Cell suspensions of Coleus were mixed with agar containing 90 mM NaCl then plated. After four weeks 1 cm diameter agar plugs containing the small cell colonies were removed from the agar and transferred to the surface of fresh agar me-

dium containing the selective agent to encourage more rapid growth of the colonies and reduce the problems of drying out.

Conner and Meredith (1985a) adopted a different procedure to the standard plating technique in selecting for aluminium resistance in *Nicotiana plumbaginifolia*. A small volume of cell suspension was dispersed over filter paper supported by polyurethane foam and saturated with medium containing aluminium (600 μM Al^{3+}, as Al_2 $(SO_4)_3$). Surviving colonies were then propagated. In a similar approach to selecting for Al tolerance, in potato cell cultures, Wersuhn *et al.* (1986) transferred cell suspensions to petri dishes containing liquid medium with filter paper to support the cells. The medium containing 2 mM Al^{3+} was replaced every seven days. Surviving callus colonies were then removed from the surface of the paper. In another selection for metal tolerance cells of the wild tomato *Lycopersicon peruvianum*, were selected for resistance to cadmium in a stepwise increase as a cell suspension culture (Bennetzen and Adams, 1984). However, in order to stimulate the limited number of surviving cells to grow out of the largely dead cell aggregates, the cells were transferred to agar medium containing the same concentration of cadmium as the liquid medium. Resistant colonies were then transferred back to liquid medium to continue the stepwise selection.

In resistant cultures derived from plated cell suspensions, stability of the trait was again confirmed by assessing its expression after a period of culture in non selective conditions. *Nicotiana tabacum* selected for resistance to thiocarbamate was only maintained for four subcultures (Flashman, 1985) whereas *Daucus carota* cultures retained their resistance to methotrexate for at least six months (Cella *et al.*, 1987) and zinc resistant cultures *Haplopapes gracilis* were stable for two years (Gilissen and Van Straveren, 1986). In none of these cases was plant regeneration reported.

Where the cell suspension cultures were only maintained for a short period as a tissue culture before a direct selection on solidified medium, then the capacity for differentiation appeared to be retained by the culture and plants could be regenerated. Thus tobacco callus selected for resistance to vernolate (Flashman *et al.*, 1985) produced resistant regenerated plants. Resistance to S-(2-aminoethyl)-cysteine (AEC) in selected callus of dihaploid potato was also shown by the regenerant plants (Jacobsen, 1986). Conner and Meredith (1985 a, b) however took the genetic analysis further. Regenerant plants of *Nicotiana plumbaginifolia* were derived from callus selected for resistance to aluminium. This resistance was found in the progeny which confirmed that the aluminium resistance was due to mutation rather than an epigenetic change.

The use of plated cells is not restricted to resistance selection. Colonies obtained in this way also provide a convenient source of material for total selection, for example to identify secondary metabolite producing lines (Chapter 8) or auxotrophs (Chapter 10). Additionally, many efforts aimed at genetic manipulation of protoplasts (Chapters 11, 12 and 13) involve late selection among the plated, protoplast-derived, colonies.

1.3.4 Protoplasts

Ideally an *in vitro* selection programme should be based on single cells since then each cell is exposed uniformly to the medium and gives rise to a discreet colony, thus avoiding the production of chimaeras. The only tissues which are truly single cells are protoplasts (Fig. 1-3); cell suspensions rarely consist solely of single cells. Although protoplasts can
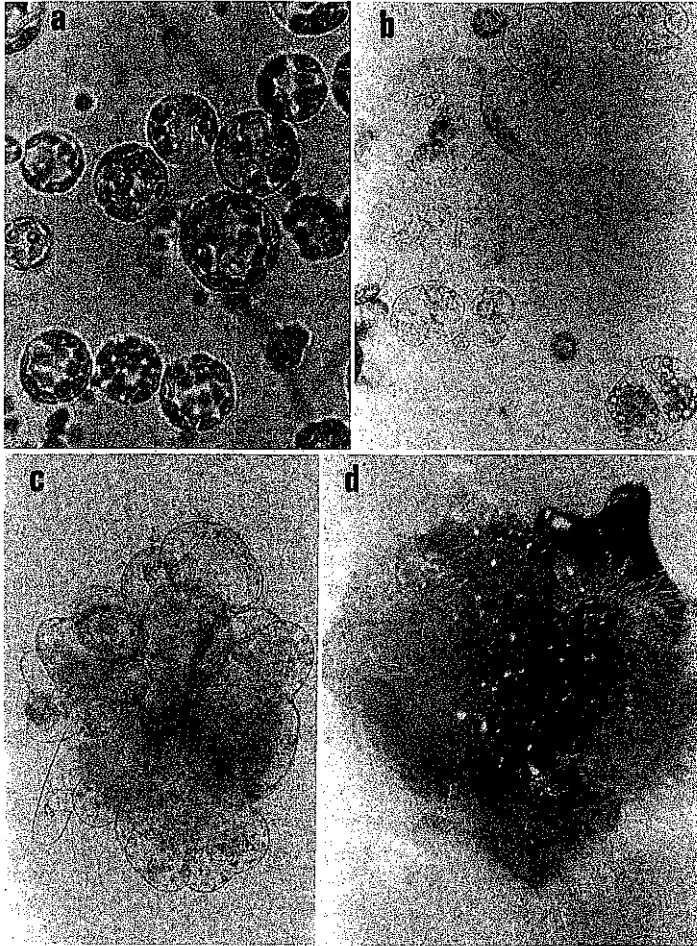
Fig. 1-3. Stages in protoplast culture, illustrated for *Brassica oleraceae* var. Botrytis (cauliflower): (a) freshly isolated mesophyll protoplast, (b) early cell divisions (7 days), (c) protoplast-derived colony (3 weeks) and (d) green, macroscopic (3 mm diameter) colony showing beginning of shoot and root differentiation (8 weeks). *In vitro* selection may be commenced at any stage, but (b)-(d) are usually preferred to freshly isolated protoplasts (photographs courtesy of C.M.O'Neill).

now be isolated from many species, such tissues are not used routinely in cell selections. The reasons for this limited attention for many species are that the yields of protoplasts are often too low, the viability of the isolated protoplasts may be variable and the procedures for initiating wall formation and division are often complex and not always successful. Direct selection at this stage adds yet another level of manipulation to an already complex procedure. These problems have not deterred some invetigators, but their efforts have largely been restricted to species for which protoplasts can easily be cultured, such as *Nicotiana* species. Selection is usually delayed until cell division has commenced, or even until the colony stage (Fig. 1-3). The more recent reports include that of Muller *et al.* (1985), who isolated protoplasts from tobacco mesophyll and induced variation in the

1 day old isolated protoplast by exposure to UV or N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG). Four days after treatment the protoplasts were plated on a medium containing the selective agent, naphthaleneacetic acid (NAA) at concentrations of 5-50 μM. Alternatively the leaves were mutagenised by exposing to ^{60}Co then the protoplasts isolated as before. Measured by the number of regenerant plants resistant to NAA, the UV and MNNG treatments were more successful than the ^{60}Co treatment. In a procedure they developed to obtain herbicide resistant mutants, Cséplö *et al.* (1985) mutagenised mesophyll protoplasts from *Nicotiana plumbaginifolia* using N-ethyl-N-nitrosourea. These were then grown up to the colony stage before being plated in medium containing the triazine herbicide. The choice of culture medium was critical as selection was dependent on the expression of the primary symptom of the herbicide action (bleaching) in the plated colonies. Selection was based on the retention of chlorophyll by the resistant colonies. Plants were regenerated and resistance shown to be inherited maternally.

In a search for temperature sensitive mutants, protoplasts were isolated from *Nicotiana plumbaginifolia* leaf mesophyll treated with UV, then plated to provide colonies for screening (Blonstein *et al.*, 1988). Marja *et al.* (1986) induced variation in leaf mesophyll protoplasts of *Hyoscyamus muticus* by exposing them to MNNG, UV or to X-rays. Protoplasts were maintained in liquid medium, then colonies of 1-2 mm diameter were plated, and subsequently analysed for scopolamine accumulation. Both these examples illustrate the use of mutagenised protoplasts to provide colonies for subsequent total selection.

Instead of inducing variation in leaf mesophyll protoplasts by mutagen treatment, other investigations have made use of variation alrady present in the cultures. Thus, Hamill *et al.* (1986) in selecting for streptomycin resistance in *Onobrychis viciifolia* isolated protoplasts from a cell suspension. The protoplasts were cultured for a period of 14-20 days to provide cell aggregates of about 30 cells which were then plated onto agar in the presence of streptomycin. Fujita *et al.* (1985) used a similar approach to stabilise the genotype and increase the yield of shikonin in cell lines of *Lithospermum erythrorhizon*. Protoplasts were isolated from cell cultures then maintained for two months to give colonies of 0.5-1.0 mm diameter. The individual cell lines which were analysed for shikonin reflected the variation found in the original source. Stable high yielding lines could, however, be selected in this way.

Not all the secondary metabolite producing lines obtained through screening protoplast-derived colonies have proved stable. For example, despite the use of mutagenic treatments, the high scopolamine yielding lines of *Hyoscyamus muticus* (Marja *et al.*, 1986) were unstable. The accumulation of the metabolite had declined to the average level by the sixth subculture, suggesting an epigenetic basis for the initial variation.

One attraction of the use of protoplasts for selection. *in vitro*, where the culture conditions are suitably well defined, is the rapidity with which one can move from selected cell back to plant. This may reduce the incidence of somaclonal variation, and certainly minimises the problem of declining morphogenetic potential frequently associated with established callus or suspension cultures. So it is that for several of the more recently reported variants (Muller *et al.*, 1985; Cséplö *et al.*, 1985; and Blonstein *et al.*, 1988) it was possible to demonstrate the transmission of the selected trait to the progeny.

1.3.5 Differentiated Cultures

Since the main purpose of *in vitro* selection is usually to produce plants with a modified genotype, a capacity to regenerate plants from the selected cells is essential. This is frequ-

ently difficult with established callus or suspension cultures, and for many species routine regeneration from protoplast-derived colonies remains problematical. One way of bypassing these difficulties is to commence selection or screening with fully or partially differentiated tissues.

An effective method for obtaining biochemical, including amino acid analogue, resistant mutants in barley involved screening large numbers of excised M_2 embryos after seed mutagenesis (eg, Bright *et al.*, 1979, Kueh and Bright, 1981). Although mutants could be produced in this way, the method is extremely laborious and only a limited number of individuals can be screened. An alternative was that developed by Merrick and Collin (1982), who used tissue culture derived embryos to screen for herbicide resistance in celery. Large numbers of embryos were produced by the differentiating cell suspension cultures, thus providing a good basis for *in vitro* screening. Since each embryo was derived from a single tissue culture cell (Dunstan, 1982), the level of somaclonal variation between the embryos provided the necessary variation. The major problem with this approach is that only a few species in tissue culture regenerate so readily and produce such large numbers of embryos. An example is Napier grass, *Pennisetum purpureum*, in which embryogenic callus was selected for salt tolerance (Chandler and Vasil, 1984). In a direct selection, callus pieces (20-25 mg) were placed on a medium containing 1.25% NaCl and subcultured on this medium regularly. Alternatively callus was transferred to medium containing 0.4% NaCl then every two weeks subcultured onto medium containing increasing concentrations of NaCl up to 2%. Plants were regenerated then tested for salt tolerance. This regeneration was initiated rapidly since prolonged subculture of the callus led to loss in regeneration. Although salt tolerant lines of embryogenic callus was obtained in this way the regenerated plants were not tolerant.

Whole plant and tissue cultures can in some cases be combined to provide an efficient protocol for the selection of mutants. Thus, Fluhr *et al.* (1985) were able to select plastome encoded antibiotic resistant mutants of tobacco by regenerating plants from resistant "green islands" on the cotyledons of M_1 seedlings, bleached by the antibiotics. The key to the success of this method was the use of an efficient plastome targetted mutagen, nitrosomethylurea, for seed mutagenesis. The same mutagen was recently used in a selection procedure involving the direct formation of adventitious shoots on leaf explants (McCabe *et al.*, 1989). Leaf strips of several solanaceous species were incubated with the mutagen and then placed on medium which normally promotes adventitious shoot production, but containing levels of streptomycin which suppressed the development of these shoots and bleaches the explant. Resistant mutants appear as green adventitious shoots (Fig. 1.4). The procedure works particularly well for *Solanum nigrum*, where spectinomycin resistant mutants have also been obtained, and lines with multiple chloroplast markers are now available.

It is in the area of selection of transformed shoots that the use of explants has really come into its own. The leaf disc transformation method, in which explants are cocultivated with strains of *Agrobacterium tumefaciens* carrying suitable resistance genes, and then incubated on regenerating medium containing the selective agent (Horsch *et al.*, 1985, see also Chapter 13) has proven an efficient procedure for obtaining transgenic plants of several species.
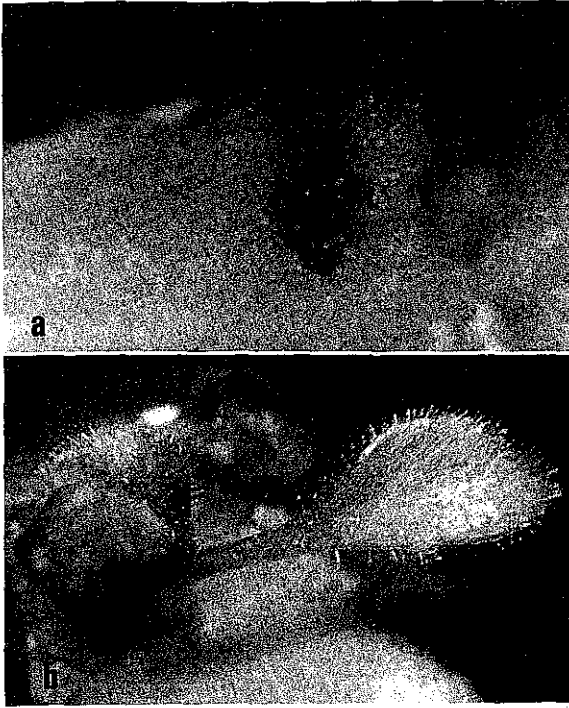
Fig. 1-4. Selection of streptomycin resistant mutants on leaf explants of *solanum nigrum.* Leaf strips treated with 5 mM nitrosomethylurea (90 min) are placed on regenerating medium containing 500 mg/L streptomycin sulphate, which suppresses adventitious shoot initiation and causes bleaching. Resistant mutants appear first as green nodules (a) which subsequently develop into green shoots (b), (photographs courtesy of P.F. McCabe).

1.4 Concluding Remarks

In plant organ, tissue or cell culture variation may arise spontaneously, or may be induced, by chemical or physical mutagens, or introduced, by cell fusion or transformation. Progress in exploiting this variation for fundamental biochemical or developmental investigations, or to achieve biotechnological objectives, is dependent on efficient methods for identifying and isolating the variant phenotype. The choice of the most effective tissue culture system for carrying out this selection is informed by a number of interrelated factors. These include the basis for the generation of the variation (eg, culture induced, mutagenesis, cell fusion or transformation), the phenotype concerned, the ultimate goal, and the culture repertoire of the species under investigation.

In this opening chapter we have attempted to put these factors into perspective, in outlining the range of selection strategies and culture systems which may be exploited. In applying this technology many problems have been encountered. These include the produc-

tion of the variation, the selection process itself, the numerous frustrations imposed by the limitations and unpredictability of the culture systems, and the stability of the selected trait, in cell cultures, regenerated plants and progeny. In addition to detailed technical consideration of the approaches to some of these problems, the remaining chapters of this volume will provide a critical analysis of the progress which has been made in applying cell line selection within a number of defined areas of study.

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