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Cell and Tissue Culture: Potentials for Plant Breeding

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Cell and tissue culture: potentials for plant breeding

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For the plant breeder, one of the objectives of cell culture systems should be their exploitation for the induction and isolation of mutant cells, which can then be regenerated as mutant plants. While a number of mutations have been recognized in plant cells *in vitro*, few have had any significance for plant breeding. There are currently a number of constraints to the exploitation of this technology, some of which are related to methodological limitations; these are likely to be overcome, but others, which relate to the nature of the attributes that the plant breeder seeks to modify, are much more intractable. There is scope for exploiting cell cutures as genetic tools, as has already been done with animal cell cultures. In contrast, the culture of organized tissues in the form of meristems or small shoots has begun to be useful a technique for plant breeders, and examples of diverse applications will be discussed. Most exploit the rapid rates of multiplication, and the assured health status of the propagules, that can be attained in culture; there is also the possibility of manipulating the genotype of these tissues. Finally, organ culture, and it is the culture of embryos that is of most interest to the plant breeder in this context, is considered; the value of embryo cultu:e as a means of producing novel interspecific and intergeneric hybrids is well recognized. In addition, cultured embryos can be used as experimental systems for studying the biochemistry and molecular biology of storage product synthesis and accumulation.

The totipotency of plant cells and the relative ease with which they can be cultured *in vitro* have engendered a degree of optimism that cell and tissue culture can provide a useful new technology for plant breeders, but in only a few instances and only for particular kinds of application has this optimism been justified. I shall discuss some of the achievements as well as the limitations of cell tissue culture, excluding from consideration pollen and protoplast cultures, as these topics are discussed elsewhere in this symposium (by Hermsen & Ramanna and by Cocking), and deal first with single-cell culture, then culture of cell groups, of organized tissues and finally of plant organs.

Cell culture

One of the objectives of cell culture systems in a plant breeding context is the induction and isolation of mutant forms and the regeneration of plants from such mutants. Single-cell culture has been successful in few instances; the production of embryoids from single carrot cells was noted by Steward *et al.* (1958), of plantlets from single cells of *Macleaya cordata* by Kohlenbach (1965), and a few other examples are known (see Narayanaswamy 1977). From an analytical point there would be advantages in being able to generate embryoids or colonies from single cells, preferably plated at low densities; there also could be other benefits in avoiding the mixture of cells of differing genotype that can occur within a group, since in these circumstances a mutant can be swamped by faster-growing wild-type cells surrounding it, or be killed by the lytic products of dying cells around it.

The culture of groups of cells growing as callus masses in liquid or on solid media has formed

the basis of most of the experimental work that has been undertaken, and the cells of a very large number of plant species can now be cultured in this manner. This system suffers from the cells' need to be grown at high densities, their tendency to genetic instability and the difficulty of regenerating plants at high frequencies. The two last problems may well be a function of the cell type that tends to occur in many callus cultures; this often consists of large highly vacuolated cells, whereas it is a general experience that small highly meristematic cells are less prone to these problems. There is an important area of research in the role of cell geometry, of the cell wall and of cell–cell relations in genetic stability and differentiation in plant cells. However, the technical problems of genetical stability and ease of differentiation will be overcome as more appropriate media and conditions are defined. It is a moot point whether another technical limitation, the inability to replica-plate plant cells, will be overcome.

Another kind of limitation has been noted in some experiments: the differential expression of characters in cells grown *in vitro* and those *in vivo*. For example, Widholm (1980) recently quoted four examples in which aspects of amino acid biosynthesis were different in cultured cells from those in the plants from which they were derived, or the plants to which they gave rise. One example was that of 5-methyltryptophan-resistant tobacco cells that had an altered anthranilate synthetase; plants regenerated from resistant lines selected *in vitro* did not show the altered enzyme, although cultures derived yet again from these plants once more had the modified enzyme. This limitation is certainly not ubiquitous, and examples of consistency of expression in cells *in vitro* and *in vivo* are known (see Maliga (1978) for references). Examples are nicotine content (Kinnersley & Dougall 1980), resistance to valine (Bourgin 1978), to picloram (Chaleff & Parsons 1978), to 5-bromodeoxyuridine (Márton & Maliga 1975) and to the fungal pathogen *Phytophthora parasitica* in tobacco (Helgeson *et al.* 1976) and *P. infestans* in potato (Behnke 1980). Two well documented examples of the exploitation of cell culture of significance to plant breeding are those in which *Pseudomonas tabaci* resistant tobacco strains (Carlson 1973) and *Helminthosporium maydis* resistant forms of *Zea mays* (Gengenbach *et al.* 1977) were produced. Attempts are also being made to select in culture for altered levels of specific constituents of crop plants, those of nicotine in tobacco (Collins & Legg 1979) and of urease in soybean (Polacco 1976) being good examples.

Although there are a few examples of potentially useful classes of mutants being selected, there are severe limitations on the kinds of selective techniques that can be exploited and thus of mutant forms that can be recognized *in vitro*. The complexity of many of the attributes that we seek to improve in our crops, and our ignorance of that which underlies them at a cellular and biochemical level, are severe constraints in this context. For example, while we can isolate disease-resistant cell lines when this is based on resistance to a pathogen-produced toxin (see Earle 1978), in the vast majority of instances we have insufficient understanding of that which underlies resistance to allow us to derive a selection régime. Such limitations to our understanding of the molecular biology of the components of plant productivity are likely to be greater barriers to progress in the near future than the technical problems of cell culture; the same is true of the techniques of genetic engineering in plants.

For those attributes that are only expressed in particular differentiated organized tissues, and they currently constitute a substantial proportion of those in which the plant breeder is interested, cell culture can at present offer us little. It has been considered by some that one exception to this might be storage proteins; it is assumed that they are only synthesized in seed. In these proteins, particular amino acids may be deficient, e.g. lysine in barley and rice, and methionine in legumes.

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It has been suggested that if mutant cells could be isolated in culture that overproduce the required amino acid, it is possible that the seed proteins might also contain more of that particular amino acid. This is a tenuous argument, and Boulter & Crocomo (1979) have stated that in legumes there is as yet no evidence that protein quality is dependent on the supply of particular amino acids. Chaleff & Carlson (1975) isolated mutant cultures of rice, on the basis of their resistance to the lysine analogue *S*-β-aminoethylcysteine, that overproduced lysine, but as no plants could be differentiated from the cultures the consequence of these mutational changes on the seed protein was not tested. More recently, Hibberd *et al.* (1980) have generated *Zea mays* lines able to grow on normally inhibitory levels of lysine plus threonine and found that the selected cultures had increased concentrations of particular amino acids; for example, in one line the lysine was twice, and in another the methionine concentration 3.8 times, as high as in the control. Only free aspartate-derived amino acids were increased. Plants were regenerated from these cultures, but no further generations could be derived and so once again the consequences in terms of seed protein could not be evaluated.

One of the problems of callus cultures, their proneness to genetic instability, has been turned to advantage in sugar cane, where variants have proved useful (Nickell 1977). Forms showing improved resistance to a virus and to fungal pathogens have been obtained; it has to be noted, however, that sugar cane is a vegetatively propagated species, with very high chromosome numbers and tolerant of chromosomal changes.

It is difficult to summarize a topic that has generated on average one symposium per year in the last few years, but while a great deal of attention has been drawn to the potential role of this technology in plant breeding, the achievements are minimal as yet. The extent to which this will alter will depend in part on our ability to improve the technology of cell culture and attain a greater expression of characters *in vitro* than is now possible. Cell cultures have not yet been exploited as genetic tools in plants, for mapping, and for complementation and linkage studies, as they have been in human cell systems, for example (McKusick & Ruddle 1977). Neither have we examined whether it is feasible to induce, and if so what might be the consequences of duplicating, certain chromosomal regions in plant cells grown *in vitro*. In mouse cell cultures, lines can be selected that show enhanced resistance to the antifolate drug methotrexate, due to increased dihydrofolate reductase activity. The resistant cells achieve this by a selective amplification of the genes for dihydrofolate reductase (Alt *et al.* 1978). DNA amplification occurs in plants *in vivo* in the well documented example of the giant chromosomes of *Nicotiana* hybrids (Gerstel & Burns 1976). Claims of DNA amplification in plant cells grown *in vitro* have been made (see Buiatti 1977), but its phenotypic consequences have not been analysed; this is an important challenge for us. It is significant that in the mouse cells, DNA other than the gene sequences directly selected was amplified (Nunberg *et al.* 1978). If that also occurred in plant cells, then genes that could not themselves be directly selected in culture, but which were closely linked to those that could, might be amplified and the consequences examined.

The culture of tissues

The ability to culture organized tissues in the form of very small shoots or meristems has allowed a most valuable application of plant tissue culture. Meristem culture has long been used for the production of virus-free plants, while the culture of small shoots and meristems is being exploited for the rapid vegetative multiplication (micropropagation) of a range of horticultural and agri-

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cultural plants (Holdgate 1977; Murashige 1978). In the context of plant breeding it is now also possible to cite many examples in which it is advantageous to exploit either the ability to propagate by tissue culture genotypes in which there is no natural or simple method of vegetative propagation, or the more rapid rates of multiplication that can be attained *in vitro*.
Such applications include the following.

(*a*) New varieties are often not available to the agricultural or horticultural industry for many years after the recognition of their value, simply because of the time taken to generate appropriate quantities for large-scale planting. Examples among vegetatively propagated horticultural crops are daffodils, freesia, gladioli and alstromeria, in all of which the natural rates of multiplication are low, and 10 or more years may elapse before a desired strain becomes available. In all of these micropropagation techniques are being used to speed up the release of new varieties to the industry. Another example in which the technique has been exploited is in the multiplication of new strains of rootstocks for top fruit.

(*b*) There is a need to maintain new varieties of vegetatively propagated plants in a disease-free condition for as long as possible during their period of multiplication before release. This means that micropropagation can be an attractive and economic alternative to conventional methods of multiplication even though a species may have a rapid rate of natural propagation. We may well find in the near future that strawberries and potatoes will be in this category.

(*c*) The breeding system of a crop plant can impose limitations on the multiplication of a genotype. In some such instances, micropropagation techniques can be a useful means of overcoming this, as the following examples illustrate.

(i) *Incompatibility systems*. In particular forms of *Brassica oleracea*, F_1 hybrids have a considerable commercial attraction; their production is dependent on the selection and maintenance of pairs of inbred parents. The sporophytic incompatibility system of these genotypes means that maintenance of the parents depends on bud pollination, which is both difficult and expensive; micropropagation offers an alternative method of maintenance and multiplication (Dunwell & Davies 1975).

(ii) *Male sterility*. Maintenance of male-sterile genotypes demands a continuous process of backcrossing, and again micropropagation can be an attractive alternative. For example, male-sterile onions can be readily propagated in tissue culture (Hussey 1978), and several male-sterile lines of wheat have been multiplied in this manner (G. Hussey, personal communication).

(iii) *Dioecious forms*. Tissue culture has been used for the clonal multiplication of selected genotypes of asparagus that are required as parental plants for the production of commercial quantities of seed (Dore 1975).

(iv) *Heterozygous genotypes*. The multiplication of large quantities of particular heterozygous genotypes to be used as parents in a seed production programme can now be achieved even though no, or only a slow, method of natural vegetative propagation exists. The onion crop again provides one example of such a species, and both diploid and tetraploid sugar beet (Hussey & Hepher 1978) may be other candidates for incorporating a micropropagation step into the breeding programme.

(*d*) Multiplication of existing superior genotypes. In some species the long life cycle or/and the heterozygosity of the plants renders conventional breeding methodology difficult; in addition, in some such instances there is no natural method of vegetative propagation. The availability of a micropropagation technique that would allow existing superior genotypes to be cloned could significantly improve the average level of performance of such a crop; a prime example in which

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clonal plantings of superior genotypes will lead to an increase in productivity is the oil palm (Jones 1974).

(*e*) Correlation of seedling and mature plant responses. Selection for disease resistance is often facilitated if it can be based on the screening of seedlings. However, their responses need not be identical with those of mature plants, and the extent to which they are correlated is not easy to establish in many instances. In homozygous forms, sister plants can be used for this comparison, but with heterozygotes this is not possible. By exploiting micropropagation, my colleagues Matthews & Dunwell (1979) were able to overcome this problem in the carnation crop; from a given seedling, the tip was taken for culture and used to generate a clone of adult plants. The response of the remainder of the seedling to a given pathogen and of the adult plant derived from the shoot tips could then be compared.

(*f*) Manipulation of the genotype.

(i) *Production of polyploid forms*. Treatment of cultures with colchicine has allowed higher rates of production of polyploids than is usually possible by more conventional techniques. In one series of experiments with freesia, which involved placing the cultures in a colchicine solution for 24 h, 27 % of the plants regenerated from a treated diploid culture became tetraploid, whereas none were found in the control (Davies 1973). High yields of tetraploid carnations have been produced in a comparable manner (Dunwell & Cornish 1978).

(ii) *Manipulation of cytoplasmic male sterility*. It has been reported that cytoplasmic male sterility in sugar beet can be 'cured' by heat treatment (Lichter 1978) and that it is graft transmissible (Curtis 1967). Sugar beet is highly heterozygous and is not readily propagated by vegetative means, but the availability of clonal material would greatly facilitate such experimental approaches. Furthermore, heat treatment of cultured tissues can be readily undertaken, as well as the grafting of propagules *in vitro*. The feasibility of manipulating male sterility in this way is currently being examined by using tissue culture, and the provision of clonal material is also aiding the comparative analysis of mitochondrial DNA in cytoplasmic male sterile and in fertile genotypes (A. Powling, personal communication).

(iii) *Analysis of genotroph induction*. The production of genotrophs in flax (Durrant 1962) is accompanied by numerous changes in the genome (Cullis 1977). The study of one of these changes, that induced in the ribosomal genes, has been facilitated by the availability of a tissue culture system (Cullis & Charlton 1981). The terminal portion of the shoot of young flax seedlings was harvested at various times after initiating the treatments that induce the genotrophs, and the ribosomal DNA (rDNA) within them assayed; the remainder of the stem below this region was then cultured, and from each of the axillary meristems that were subsequently induced to develop, the rDNA was extracted and assayed. In this way Cullis & Charlton could determine when the changes occurred in the rDNA during the process of induction. They showed that the changes occurred rapidly and only in the terminal regions of the stem, an analysis that would otherwise be extremely difficult to achieve.

(iv) *Induction of mutations*. Adventitious meristems can develop from single cells; if these cells have been modified by exposure to mutagenic agents, wholly mutant meristems and plants may be immediately generated. In such meristems the competition that occurs between wild-type and mutant cells, which can result in the suppression of the latter, is avoided. The end result should be a higher rate of recovery of mutant plants. Adventitious buds are readily generated in culture, and as Broertjes *et al.* (1976) have shown with chrysanthemum, they can be a useful source of induced mutations.

(*g*) Storage of genotypes. The maintenance of selected heterozygous lines to be used as parents

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in the production of commercial varieties can often be difficult. If they are maintained by seed multiplication, there is a danger of the occurrence of genetic drift. Storage of vegetatively propagated material can equally be difficult. Tissue cultures can, however, be stored for many months and even years in some instances, simply by keeping them on a nutrient agar medium at 4 °C and at low light intensities. This offers an easy, cheap and, furthermore, disease-free system of maintaining parental lines for variety production.

(*h*) Provision of disease-free material. Disease-free strains are required for an unbiased evaluation of potential new varieties and also for the selection of parents. Dale (1975) has suggested that the breeding of certain grass species in which virus infection rapidly leads to a marked reduction in plant vigour would be aided by the availability of disease-free forms.

These examples illustrate the opportunities that are already available to the plant breeder to exploit the culture of organized tissues, but a further expansion of the technology will undoubtedly occur as the culture of a wider range of species and genera becomes possible, and as plant breeders recognize the role it can play.

Organ culture

For the plant breeder it is the culture of embryos that is of primary interest in this context. The production of many interspecific hybrids has been possible as the result of the rescue of immature embryos by excision from the ovary and their subsequent culture *in vitro*. A recent review (Raghavan 1977) has summarized the applications of embryo culture and the range of hybrids produced in this manner. Included among them are intergeneric hybrids involving *Triticum* and related genera, and *Zea mays* and its relatives, as well as interspecific crosses involving *Triticum*, *Hordeum*, *Oryza*, *Sorghum*, *Nicotiana* and *Hordeum*, to list but a few of many important crop plants. While it is unlikely that such hybrids themselves or their allopolyploid derivatives will be useful as crop plants, they do offer a means of achieving an interspecific or intergeneric transfer of chromosomes or of chromosome fragments. For this latter purpose they can sometimes offer an easier alternative to the route offered by protoplast fusion. The interspecific and intergeneric embryos have the advantage that nuclear fusion, cell wall formation and initial cell divisions have already been achieved. The elimination of particular chromosomes in interspecific embryos can occur naturally, as in the hybrids of *Hordeum vulgare* and *H. bulbosum*, and of *Nicotiana plumbaginifolia* and *N. tabacum*, leading in these instances to the production of offspring in which only one parental genome is present; this has been the basis of the large-scale production by embryo culture of barley haploids (Kasha 1974). Until more effort is devoted to establishing whether the phenomenon of chromosome elimination occurs in other interspecific and intergeneric embryos, it is impossible to speculate on the wider applicability of this technology of producing haploids. A method of attaining an interspecific transfer of chromosome fragments has been developed by Pandey (1980), in which pollen is exposed to high doses of ionizing radiation to fragment the chromosomes, before its use for pollination. Fragments of the paternal chromosomes are then incorporated into, or attached to, the maternal chromosomes within the embryo. These experiments have not involved embryo culture, and few progeny incorporating alien chromosome fragments have been produced. My colleague, J. M. Dunwell, is attempting to modify Pandey's approach by inducing a proliferation of the cultured embryos produced after interspecific or intergeneric pollination with irradiated pollen; in this way he hopes to generate from each embryo a number of derivatives, each of which will have the maternal genome and also a different

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paternal fragment(s). This may enable us to sample a greater range of the fragments that can be included in the embryos.

Few attempts have been made to culture plant embryos for experimental purposes other than the generation of hybrids. This is in marked contrast to animal embryology, which has a long tradition of experimental work. In many crop plants the embryo is the economically important component, yet we know singularly little about it in cellular, biochemical or molecular terms. Embryo culture could be useful in this respect and I shall describe some of our own work on peas (*Pisum sativum*) to illustrate this. Culture methods have improved to an extent that comparable growth rates can be achieved *in vivo* and *in vitro* in *Phaseolus vulgaris* (Thompson *et al.* 1977) and in peas (Stafford & Davies 1979), although in both species the period of growth over which these rates can be maintained is still relatively limited. Mature legume seed is composed almost entirely of the embryo, with the swollen cotyledons composing the storage tissue. The number and size of cells within the cotyledons are the determinants of seed size, and while genetic variation exists for both components (Davies 1975, 1977) we do not have at present much knowledge of their respective importance. The DNA in the cotyledon cells is highly endoreduplicated, the extent of DNA duplication being proportional to cell size (Davies 1977). By an appropriate culture technique we can trigger these cells, which normally remain in interphase, into a prophase stage, in which giant polytene-like chromosomes are seen (Marks & Davies 1979). Embryo culture could be used to examine the way in which factors influencing seed size affect the two components, cell size and cell number. It could also be used to study the control of storage product accumulation within the seed. We have shown that comparable amounts of protein and starch are synthesized *in vitro* and *in vivo* in peas (Stafford & Davies 1979). Beyond this we have examined the synthesis of legumin, one of the two main storage proteins in peas, in cultured embryos, and compared it with that occurring *in vivo*. Earlier work had suggested that legumin was synthesized at a fairly late stage of development of the embryo, when the greater proportion of the cells of the cotyledon were becoming endoreduplicated; secondly, it was believed that legumin synthesis could not be initiated in cultured embryos (Millerd *et al.* 1975). By using a more sensitive assay for legumin, an enzyme-linked immunoabsorbent assay (ELISA), which allows us to detect nanogram quantities of the protein (Domoney *et al.* 1980), we have shown that legumin is synthesized in much younger embryos than hitherto assumed, an observation to which I will return later. Secondly, with improved culture techniques we have demonstrated that legumin synthesis can be initiated *in vitro* (Domoney *et al.* 1980).

Returning to the observation of the presence of legumin in the cells of very young embryos, this implies either that there is a low rate of synthesis even in the diploid cells of the young cotyledon or that there are already a few endoreduplicated cells present, and it is these that are synthesizing the protein. Should the former be true, it is important to test whether other diploid cells within the plant, and even cells in culture, can synthesize legumin, albeit at a very low level, but levels that we might now detect with the ELISA technique. It has been suggested that callus cells derived from cultures of *Vicia* cotyledons can synthesize low levels of storage protein (Muntz, quoted in Boulter & Crocomo 1979). The possibility of selecting mutant cells in culture that can overproduce particular storage proteins is attractive, and the aim in peas would be to enhance the production of legumin, which has a higher proportion of sulphur amino acids than some of the other seed proteins.

Embryo culture is being used also for studying another aspect of storage product synthesis in peas. It has been recently shown (Davies 1980) that there are mutants in peas, somewhat akin to

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those in maize and barley, in which both protein and carbohydrate composition is altered within the seed. In peas the two seed phenotypes, round and wrinkled, differ in starch quantity and quality, sugar content and storage protein composition, the proportion of legumin being higher in round seed (Davies 1980). The nature of the metabolic changes induced by the allelic alternatives at the r_a locus, which is involved in the determination of these two phenotypes, is not known, but we are using embryo culture to examine the proteins synthesized by these two genotypes when grown under various conditions to try to analyse the relation of carbohydrate and protein synthesis, and how it may be manipulated. We therefore have mutants in peas that affect storage product composition, we can culture the embryos in which these products are synthesized and stored, and we can define the ways in which we need to improve the phenotype. An important limitation, however, is the dearth of knowledge of the biochemistry and molecular biology of these important components of economic yield – the seed – and this needs to be remedied if we are to successfully manipulate and modify them by plant breeding.

Conclusion

A cautious optimism may be an appropriate conclusion; after all, plant breeders and geneticists took little interest in cell, tissue and organ culture until recently, but already the value of meristem and shoot culture is widely recognized. Other aspects of the subject will prove attractive as techniques improve and new applications are recognized, and if genetic engineering is to contribute to plant improvement, it will be mediated to a substantial extent through the manipulation of cells in culture.

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Discussion

S. Bright (*Rothamsted Experimental Station, Harpenden, Herts, U.K.*). Some recent work on plant mutants that accumulate amino acids is relevant here after Professor Davies's paper in which he questioned whether accumulation would be expressed in seeds. Complex diploid tissue cultures of maize (Hibberd *et al.* 1980) or mature embryos of diploid barley have been used to select mutants resistant to lysine plus threonine (Bright *et al.* 1981). In both cases, threonine and methionine are accumulated in the growing tissues. One barley mutant, Rothamsted 2501, contains a single dominant gene for resistance (associated with recessive lethality). Normal seeds contain little soluble threonine (less than 1 % of total threonine) whereas seeds from resistant plants have 15 % of the total threonine in the soluble fraction. This is sufficient to change the total threonine content. There is evidence for increased total methionine also in the barley mutant described above: as soluble methionine is very low this must be in protein.

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