2
Cell and Tissue Culture Technology for the Genetic Manipulation of Temperate Fruit Trees

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Introduction

Temperate fruit trees may conveniently be divided into three groups:

1. Pome fruits—apple, pear, quince (*Malus, Pyrus, Cydonia* spp.);
2. Stone fruits—peaches, plums, apricots, cherries (*Prunus* spp.);
3. Others—persimmon (*Diospyros virginiana* L.), papaw (*Asiminia triloba* (L.) Dun.), serviceberry (*Amelanchier* spp.).

This does not include citrus, which is not normally considered to be a temperate fruit crop.

These species are probably among the most suitable crop plants for genetic improvement by somatic methods. They invariably have a long juvenile phase and long reproductive cycles with concomitantly lengthy breeding programmes; they are nearly always highly heterozygous, outbreeding species and are asexually propagated. Steward in 1970 was already emphasizing the part that tissue culture might play in the genetic improvement of tree species when he noted that '. . .those who breed plants whose growth cycle is long, like trees, are already looking to cell and culture techniques to speed up their work'. For these reasons there are great benefits to be derived from rapid methods. Biochemical and physiological selection procedures applied to genetically manipulated tissue cultures appear to be particularly promising.

An added complication from both the breeding and propagation standpoints is that most fruit trees are of a composite nature consisting of the

Abbreviations: BA, 6-benzyladenine; DNA, deoxyribonucleic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; EMS, ethane methyl sulphonate; EPSP, 5-*enol*-pyruvylshikimate-3-phosphate; IBA, indolyl-3-butyric acid; NAA, 1-naphthylacetic acid; Ri, root-inducing plasmid; Ti, tumour-inducing plasmid

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fruit-producing scion grafted on to a suitable rootstock to give desirable size control and cropping effects. Both are often selected on the basis of different soil and climatic conditions, in such a way that a given scion variety of one fruit tree may assume great importance in one region but be totally unsuited for another. This is less true of rootstocks, which have more universal use, but again specific requirements may lead to the rejection of internationally known, clonally propagated rootstocks, in favour of locally grown seedling ones.

All these factors make temperate fruit tree production an expensive, labour-intensive, long-term and highly skilled operation. However, the value of these crops in global terms is considerable and any procedures that can speed up the genetic improvement and production of suitable-sized, hardy and high-yielding varieties must be welcomed.

ECONOMIC IMPORTANCE

International trade in fresh temperate tree fruits rivals that of other major non-cereal crops of the world (*Table 1*). The economic value of apple exports alone is comparable to that of the pulses and greater than that of potatoes and tomatoes.

Table 1. World values* of temperate tree fruits compared with the major crops (1000's $)

Crop	Imports	Ratio	Exports	Ratio†
Temperate fruit trees (apples, pears, peaches‡	2 176 896	1·00	1 799 950	1·00
Apples	1 477 245	0·67	1 201 293	0·66
Grapes	766 535	0·35	695 150	0·38
Potatoes	1 120 778	0·51	1 002 371	0·55
Pulses	1 412 803	0·64	1 226 898	0·68
Tomatoes	1 111 271	0·51	991 694	0·55
All cereals	41 947 510	19·26	37 453 640	20·81

* Figures drawn from FAO Production Year Book 1984
† Ratio = value of imports/exports of each group divided by value of 'temperate fruit trees'.
‡ Figures not available for apricots, plums, cherries.

As a group, temperate tree fruits have been stable in their production in recent years, with apples being by far the most prominent and exceeding the next most highly produced group—pears—by a factor of more than four (*Table 2*). In fact, apples account for approximately 60% of the total production of all temperate tree fruits and nearly 50% of all deciduous tree fruits (Brown, 1975).

PRESENT AND FUTURE STRATEGIES FOR GENETIC IMPROVEMENT

Conventional breeding methods have produced rootstocks and scion varieties with a great variety of horticultural characteristics suited to a range of

Table 2. World production* of different temperate tree fruits† (1000's metric tonnes) using apricot as standard

Crop	1982	1983	1984	Ratio‡ ('84 figures)
Apples	41 440	39 349	40 378	22·30
Pears	9 130	9 544	9 092	5·03
Peaches	7 231	7 362	7 697	4·26
Plums	6 476	6 353	6 087	3·37
Apricots	1 773	1 913	1 806	1·00

* Figures for cherries not available.
† Figures drawn from FAO Production Yearbook 1984.
‡ Ratio = production figures of each tree fruit divided by production figures of apricot.

different environments. However, a large number of existing cultivars arose as chance seedlings and have been propagated asexually for decades. Many commercial cultivars could be greatly improved in terms of fruit quality and quantity, rootability, dwarfing capacity, disease resistance, cold tolerance, drought resistance and other characteristics.

Conventional breeding techniques will not produce the major improvements sought without long-term selection and lengthy back-crossing procedures that will normally exceed the life-span of the breeder. The use of tissue culture holds promise of rapid selection for desirable biochemical/physiological characteristics (if they can be identified) and so would enable large numbers of mutants/recombinants to be examined within a short time and limited space. Already, success at regenerating plants from tissue culture with other heterozygous and asexually propagated crops such as potato (Karp and Bright, 1985; *see also* Chapter 1 of this volume) has shown that existing commercial cultivars can be improved using cell and tissue culture technology. Improvements have included increased disease resistance, changed photoperiodic responses and altered habit and leaf morphology.

It is in adding these 'fine' adjustments to existing cultivars that the newly emerging science of somatic cell genetics coupled with improved tissue culture methodology is likely to make a great impact in fruit tree improvement in the future. This review will examine how the many different facets of developed and developing cell and tissue culture technology may make this possible.

First the techniques of micropropagation are discussed briefly, as they have a crucial role in genetic improvement procedures.

Micropropagation of temperate fruit trees

PROCEDURES

Temperate fruit tree micropropagation was pioneered by Abbott and Whiteley (1976) and Jones (1976) who developed the procedures for apple shoots.

This has subsequently been extended to a range of temperate fruits (*see* e.g. Zimmerman, 1983). The technique for apple is similar to that for many other species and is based on the hormonal stimulation of multiple axillary shoot development from pre-existing organized axillary buds either as very small excised meristems or more often from surface-disinfested actively growing shoot tips 0·5–1·0 cm in length. Multiple shoots are usually induced by inclusion of cytokinins (e.g. 6-benzyladenine) in the medium (*Figure 1*). Single excised shoots are then stimulated to root by removing the cytokinin and adding auxin and possibly cofactors (Jones and Hatfield, 1976; James and Thurbon, 1981). Micropropagation by this procedure has been reviewed by Hutchinson (1981) and by Jones (1983).

The technique normally yields genetically identical cloned shoots, if formed from pre-existing organized tissues, i.e. axillary buds, without a callus phase. Many thousands of temperate fruit plants and numerous other species have been micropropagated to form clones in this way (Constantine, 1986; Harper and Fordyce, 1986), without appearance of the genomic or chromosomal modification found in plants derived from disorganized sytems such as callus (D'Amato, 1977). No matter how variants are obtained, the ability to form clones is essential if any desirable mutants are to be multiplied rapidly.

The high rates of shoot multiplication that can be obtained with micropropagation in many fruit trees have, nevertheless, not led to the widespread commercial adoption of the process. This is possibly because tissue culture technology can be expensive and costs per unit product can be high. Problems relating to commercial success or failure of micropropagated plant species have been reviewed by Constantine (1986). Apart from costs, there may not have been time to test adequately the commercial value of fruit trees derived by micropropagation. The peach × almond hybrid rootstock GF677 (Zucchcrelli, 1979) is, however, a notable exception as it is now micropropagated on a large scale in several European countries and has made a major contribution to the peach industry in Europe. Unofficial estimates (E. Rugini, personal communication) suggest that almost 10 million plants per year are produced, almost half of these being in Italy and the remainder in France and Spain. Other rootstocks such as Colt (cherry) are micropropagated commercially but on a much smaller scale.

THE IMPORTANCE OF MICROPROPAGATION AND REGENERATION IN THE GENETIC MANIPULATION PROCESS

Micropropagation systems with high multiplication rates are not only valuable in improving the propagation process, they must also form the basis for the introduction of desired and controllable genetic variation. In all cases it is necessary to be able to regenerate viable propagatable shoots either by organogenesis or somatic embryogenesis from explants taken from *in vitro* cultures. Regeneration of plants from single cells and complex explants is therefore the key process in all genetic manipulation work; unless this can be achieved consistently and efficiently, no genetic improvement by somatic methods is possible. To achieve this, a source of genetically homogeneous

cells and tissues is necessary. Micropropagating cultures are an ideal source and leaves are probably the most abundant and suitable. As all temperate fruit trees are amenable to micropropagation (*see* e.g. Zimmerman, 1983) the way is open to use them for the introduction of variation through the methods discussed below.

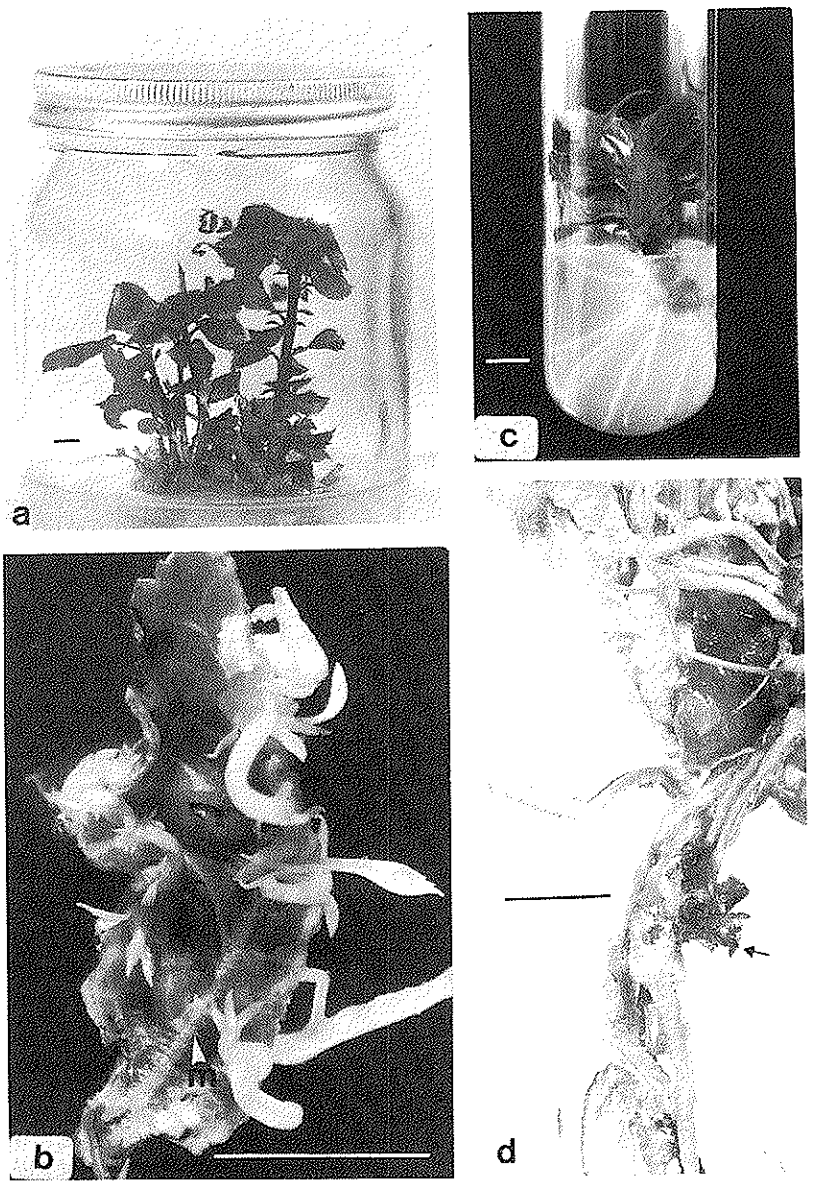

Figure 1. (a) Apple shoot culture used for regeneration and transformation work. (b) Shoot regeneration from apple leaf tissues (m), mid rib (arrowed). (c) Rooted shoot culture of cherry rootstock, Colt. (d) Shoot regeneration (arrowed) occurring from root callus (cherry). Bar = 0·5 cm.

INDUCING VARIATION

Methods for introducing variation can be either undirected or directed. Undirected variation is that which is induced artificially but without control. Variation may be wide, narrow or absent depending on the particular crop.

Directed variation refers to the introduction of foreign genes, DNA fragments, oligonucleotides or chromosomes into host cells. These processes frequently employ tissue culture methods and commonly use vectors such as *Agrobacterium tumefaciens* and *A. rhizogenes*.

For the purposes of this review the following methods of inducing variation are considered:

1. Regeneration from complex explants for the introduction of somaclonal and gametoclonal variation;
2. *In vitro* mutagenesis followed by regeneration of plants;
3. Ploidy manipulations to change chromosome number;
4. Protoplast technology;
5. Genetic transformation using *Agrobacteria*.

Regeneration from complex explants: somaclonal and gametoclonal variation

Regenerating plants from this source means that somaclonal variation can be exploited to produce improved variant cultivars that may be of immediate practical and/or breeding value. The phenomenon has been covered in numerous reviews (Larkin and Scowcroft, 1981, 1983) and may be defined as the variation observed in plants that have been regenerated from *in vitro* cultured cells, protoplasts or complex explants such as leaves, stems and roots. Usually a callus phase precedes the regeneration of shoots or somatic embryos.

For many species, somaclonal variation is already acknowledged as another aspect of breeding and full field trials have been carried out to assess its value. Scowcroft and Larkin (1982) have reviewed the process as it relates to a number of crops including potato, sugar-cane, tobacco and rice. Perhaps not surprisingly there is no mention of fruit trees. In many cases the plants are regenerated from complex explants, although the term somaclonal variation was initially coined after Shepard, Bidney and Shahin (1980) had regenerated many variant lines from potato protoplasts and called them 'protoclones'.

Fruit trees and woody plants generally are only just beginning to be 'bred' in these ways. When exploiting undirected variation in fruit trees there are four main tissues that can be considered as source material for the regeneration of plants:

1. Mature tissues from existing clonal varieties, e.g. leaves, roots;
2. Juvenile but maternal tissues such as the nucellus;
3. Unreduced megaspore mother cells that produce embryos by natural or induced apomixis (reproduction by seed without fertilization);

4. Embryonic or seedling tissues produced by normal sexual fertilization processes.

All these will have their uses, depending upon the aim of the investigator but, if improvement of existing cultivars is the prime concern, tissues that have not been involved in natural gene recombination or segregation are to be preferred.

REGENERATION FROM MATURE TISSUES OF CLONAL VARIETIES

A recent comprehensive review by Skirvin *et al.* (1986) for apple shows that no adventitious shoot regeneration was observed from tissues of non-seedling origin in the work of 35 different publications. Although in many cases the authors cited were not attempting to induce regeneration, this demonstrates that, until very recently, regeneration from mature tissues was indeed a rare event and regarded as extremely difficult, probably because tissues derived from mature woody subjects have traditionally given considerable problems with disinfestation (removal of all pests), phenolics and exudates generally, when attempts were made to introduce them into culture (*see* e.g. Smith and McCown, 1982/3).

Nevertheless, in 1979 Wei-lun and co-workers (Wei-lun *et al.*, 1979) reported shoot regeneration from callus derived from mature tissues of the apple rootstock M.9. It was in attempting to repeat and extend this work that James, Passey and Malhotra (1984) reported shoot regeneration from leaf discs and internode callus of the apple rootstocks M.25 and M.27 and the cherry rootstock Colt. Druart (1980) had previously shown that regeneration of plantlets from *in vitro** roots of several *Prunus* spp. was possible, while Jones, Gayner and Watkins (1984) repeated and extended this work by regenerating shoots from root callus of the apple rootstock M.25 and the cherry rootstock, Colt. The recent (1986) Moet:Hennessy Conference on 'Fruit Tree Biotechnology' showed that the situation has since changed considerably: there are now many reports of shoot regeneration and somatic embryogenesis from leaf and root tissues of an extensive range of temperate fruit tree genotypes of existing commercial cultivars; most of these are shown in *Table 3*. It is clear from this Table that BA plus NAA seems to be an effective combination across a wide selection of species and cultivars although other combinations of growth regulators are also effective. Generally, BA seems to be effective at 1·0–2·0 mg/l and NAA at 0·1–0·5 mg/l although more precise definition may be necessary for any given cultivar. Leaf tissues seem to be preferable as source material for *Malus*. *Pyrus* can be made to regenerate from either roots or leaves, albeit at a generally lower frequency, while root tissues have mainly been used for *Prunus* species

* In this review, *in vitro* means separated from the host plant and kept in aseptic culture (or any other culture, e.g. as a cutting or a graft); *in vivo* means the plant part or whole is living independently and 'normally', and is not subjected to the specialized environment of plants grown *in vitro*.

Table 3. Regeneration from complex explants of non-seedling and non-embryonic origin in temperate fruit tree species

Species and cultivar	Explant	Regeneration		PGRs	Reference
		Type	%*		
Apple					
Greensleeves	L	S	90	BA/NAA	James, Passey and Rugini, 1987
Tuscan	L	S	65		
Akero	L	S,E	NS		Welander, 1986
McIntosh	L	S,E	NS	BA/NAA	Welander, 1986
Wijcik	L	S,E	NS	Z/NAA	Welander, 1986
Gravenstein	L	S,E	NS		Welander, 1986
Jonagold	L	S	10		Tapia y Figueroa and Viseur, 1986
Jubile	L	S	10	BA/NAA	Tapia y Figueroa and Viseur, 1986
Degollune	L	S	10		Tapia y Figueroa and Viseur, 1986
Golden Delicious	L	S	10		Tapia y Figueroa and Viseur, 1986
Empire	R	S	NS	BA/IBA	Dufour and Zimmerman, 1986
Gala	L	S	NS	BA/NAA /IBA	
M.9	SI	S	50	BA/NAA	Wei-lun *et al.*, 1979
M.9	L	S	20	BA/NAA	James, Passey and Rugini, 1987
M.25	L	S	60	BA/NAA	James, Passey and Rugini, 1987
M.25	SI	S	30	BA	James, Passey and Malhotra, 1984
M.25	R	S	3	BA/NAA	Jones, Gayner and Watkins, 1984
M.26	L	Ed	10	BA/IBA	James, Passey and Malhotra, 1984
M.26	L	S	75	BA/NAA	Morgan, 1987†
M.26	L	S	20	BA/NAA	Tapia y Figueroa and Viseur, 1986
M.26	L	S	100	BA/NAA	Rosati and Predieri, 1986†
M.26	L	S,E	NS	BA/NAA Z/NAA	Welander, 1986

contd

(*Figure 1*), although Matsuta, Hirabayashi and Akihama (1983) were able to regenerate plantlets from leaf callus of *Prunus lannesiana*.

This upsurge in regeneration protocols marks something of a turning point for the genetic engineering of fruit trees and the way is now open for the production of a whole new generation of novel biotypes based on current commercially important cultivars.

Nevertheless, the variation in regeneration efficiency between cultivars is considerable in all plant species (*see* e.g. Ammirato, 1986). An understanding of the factors controlling regeneration is vital in developing gene transfer technology for all crop plants.

Table 3 (*contd*)

Species and cultivar	Explant	Regeneration		PGRs	Reference
		Type	%*		
M.27	L	S	10	BA/2,4D	James, Passey and Malhotra, 1984
M.27	L	S	50	BA/NAA	James, Murphy and Passey, 1985
M.27	R	S	60	BA/NAA	Yehia, Jones and Browning, 1985
MM.106	L	S	25	BA/NAA	Rosati and Predieri, 1986†
Pear					
Conference	L	S	20	BA/NAA	Rosati and Predieri, 1986†
Conference	L	S	20	BA/NAA	Morgan, 1987†
Durondeau	SI	S	NS	NS	Viseur, 1986
	R	S	NS	NS	
P661	R	S	<1	NS	Yehia, Jones and Browning, 1985
P657	R	S	<1	NS	Yehia, Jones and Browning, 1985
P658	R	S	10	NS	Yehia, Jones and Browning, 1985
Cherry					
Colt	SI	S	22	BA/NAA	James, Passey and Malhotra, 1984
Colt	R	S,E	13	BA/NAA	James, MacKenzie and Malhotra, 1987
Colt	R	S,E	25	BA/NAA	Jones, Gayner and Watkins, 1984
Plum					
Quetsche	R	S,E	50	BA/IBA GA3	Druart, 1980

*% Regeneration = percentage of explants giving rise to shoot buds or somatic embryos.
† Unpublished observations.
Abbreviations: S, shoot buds; E, somatic embryos; Ed, embryoid; L, leaves; R, roots; SI, shoot internode; NS, not stated; PGRs, plant growth regulators used; BA, 6-benzyladenine; Z, zeatin riboside; NAA, 1-naphthylacetic acid; IBA, indolyl-3-butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Welander (1986) observed embryo-like structures on apple leaf pieces, although no other reports have shown this (*Table 3*). As BA and NAA were the growth regulators used in all this work, the only difference was in Welander's use of the basal medium N6 (Chu, 1978) rather than the MS medium used by most workers (Murashige and Skoog, 1962). The N6 medium was originally used for rice anther culture and is both quantitatively and qualitatively different from MS medium in its nitrogen source. Comparative studies are needed on the effects of these media in controlling embryogenesis as opposed to organogenesis.

It is far too early to assess the effect of somaclonal variation on genetic

improvement of fruit tree cultivars but there are many possible characters that could be improved by this technique. Hammerschlag (1986) used the technique to produce variants of the peach cultivar Sunhigh resistant to bacterial spot (*Xanthomonas campestris* pv. *pruni*). Toxic metabolites produced by the bacterium were used to challenge callus cultures obtained from immature embryos. Plants regenerated from resistant cultures were reported to be more resistant than other known resistant cultivars. Similarly, Viseur (1986) has regenerated pear shoots from callus of cv. Durondeau that were resistant to the fireblight pathogen *Erwinia amylovora*. In this case, callus cultures were not challenged with the pathogen or any of its metabolites. C. R. Morgan (personal communication) has regenerated many clones of apple after challenging leaf tissues with live cells of a virulent strain of the same pathogen. Cloned versions of these plants are being assessed for resistance after transfer to soil culture.

In all work of this sort, *in vitro* resistance must be correlated with field resistance; such tests have not yet been reported.

JUVENILE MATERNAL TISSUES—APOMIXIS AND THE PROSPECT OF SOMATIC EMBRYOGENESIS

The nucellus is an ephemeral tissue and usually degenerates during embryo and seed development as the endosperm matures. In *Citrus* species this tissue has proved to be very useful in tissue culture studies where the type of apomixis known as nucellar embryony often leads to the production of maternal and true-to-type apomictic seedlings that have successfully been used as clonal rootstocks (Button and Kochba, 1977). Embryogenic callus derived from the nucellus has remained totipotent for many years and was used as source material for the first successful regeneration of a tree species from protoplasts (Vardi, Spiegel-Roy and Galun, 1982). In *Citrus* the nucellus is unique in that nucellar embryos are known to be produced from single cells (Esan, 1973), thus facilitating the production of non-chimaeral mutants.

These advantages have led other workers to emulate this approach with temperate fruit trees. However, of these only the genus *Malus* is believed to show apomixis, which is of the apospory type, i.e. the development of somatic cells into a gametophyte but with unreduced gametes (Dermen, 1936). There are reports of adventitious embryos from the nucellus of apple (Eicholtz, Robitaille and Hasegawa, 1979; James, Passey and Deeming, 1984), pear (Janick, 1982) and from mango (Litz, 1985).

James and co-workers (James *et al.*, 1986a,b), using several apple cultivars, produced large numbers of adventitious embryos which could be cloned by micropropagation (*Figure 2*) and weaned into functional plants. Unfortunately, the exact origin of these embryos could not be ascertained, because of the close proximity of the nucellus, integuments and endosperm at the micropyle at the time of excision from the immature seed. The adventitious embryos arose from within the ovular tissues only after the presumptive zygotic embryo had been removed and the remaining nucellar tissues cultured (*Figure 2*). To verify the origin of these plants it was necessary to repeat the

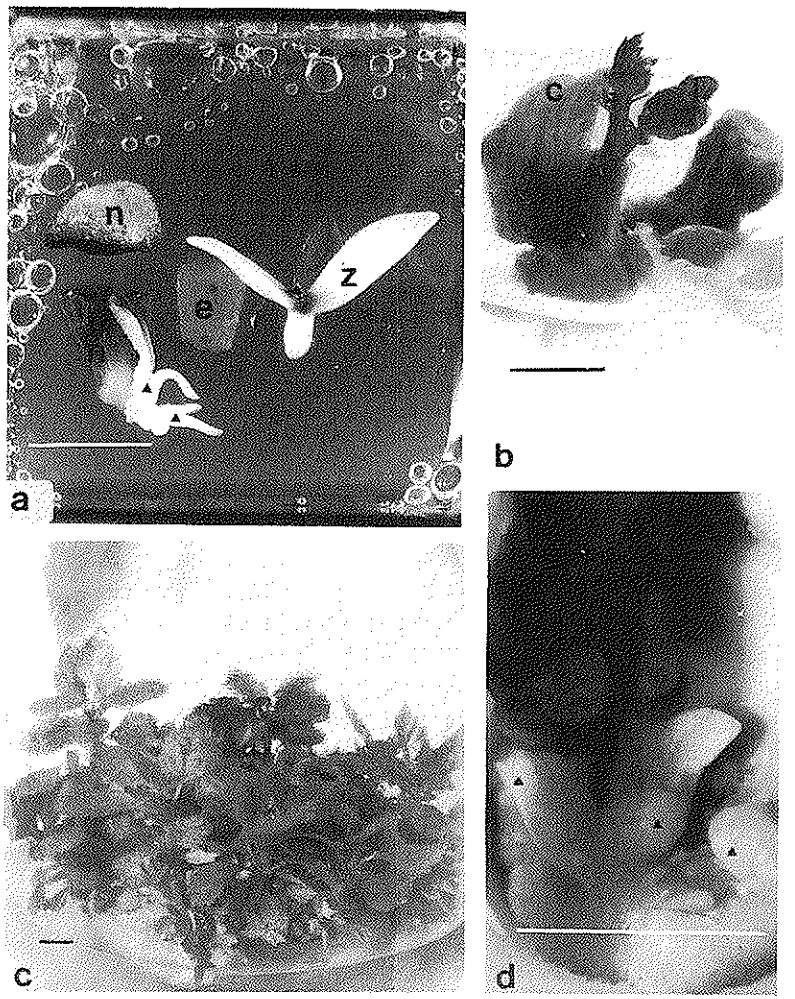

Figure 2. Adventitious embryogenesis in apple. (a) Adventitious embryos (▲) attached to the nucellus (n). e, endosperm; z, zygotic embryo. (b) 'Germinated' adventitious embryo; c, cotyledon; l, first true leaf. (c) Micropropagated culture from a single adventitious embryo. (d) Secondary adventitious embryos (▲) growing on the cotyledonary surface of zygotic embryo. Bar = 0·5 cm.

previous work, which was based on open-pollinated crosses, by using apple pollen carrying a dominant, homozygous marker gene, *R*, for anthocyanin formation. This gene is carried by the apple cv. Baskatong (Church and Williams, 1978) and is expressed in all progeny and in all tissues of zygotic origin.

When these controlled crosses were performed, adventitious embryos again formed from dissected nucelli. However, when these were micropropagated, anthocyanin pigmentation developed in stem and leaf tissues *in vitro* in all three cultivars examined and in over 200 different embryos from all

three cultivars used, thereby proving that these embryos were of zygotic origin and thus not clones of the donor tree. Although adventitious embryos can be seen to be distinct from the true embryo (*Figure 2*), microscopical evidence (E. C. M. Menhinick, unpublished data) shows that they are probably derived from zygotic cells or groups of cells that have been removed inadvertently from the zygotic embryo during the dissection of the seed and transferred with the nucellus and endosperm. They later express their regenerative capacity during the following weeks in culture.

These observations have important implications for the use of somatic embryogenesis as a means of propagation employing the nucellus as starting material. If a suitable pollen marker is not available the nucellus should not be used. Reports of regeneration from nucellar tissues in any crop where a pollen marker is not used are thus equivocal. The absence of pollen marker genes in many tree species could be overcome by formation of haploids followed by production from them of fertile dihaploids. The possibility of inducing somatic embryogenesis in fruit trees from maternal tissues should not be dismissed. As previously pointed out, it has already been shown that embryoid-like structures can arise on the surface of non-seedling apple leaves (James, Passey and Deeming, 1984; Welander, 1986) and of plum and cherry roots (Druart, 1980; Jones, Gayner and Watkins, 1984). These arise in static culture only; to convert this to a system of mass propagation of thousands of tiny somatic embryos in a liquid batch culture system similar to that devised for carrot and other herbaceous species (Redenbaugh *et al.*, 1986) will require considerable increases in propagation rates. Some encouragement may, however, be drawn from the fact that in cherry cultures embryoid-like structures have been converted into normal-looking plants after micropropagation and transfer to soil (James, MacKenzie and Malhotra, 1987), with no obvious phenotypic variation from control plants.

There are no reports of nucellus culture in any of the other temperate fruit tree crops. Possible use of the endosperm to produce triploids is dealt with in the section on ploidy manipulation.

REGENERATION FROM MEGASPORE MOTHER CELLS VIA INDUCED APOMIXIS

Natural apomixis in apple can lead to the production of embryos genetically identical to the mother plant. Such embryos develop from functional unreduced gametes. Campbell and Wilson (1962) realized some years ago that this phenomenon could be exploited to produce seedling rootstocks that would impart uniform growth to the grafted scion and be virus free. The subjects chosen were all triploid *Malus* species, where according to Dermen (1936) the embryos had a nucellar origin, the true embryo sac having degenerated.

Although this situation is not widespread among apple cultivars, it demonstrates the possibility of inducing parthenogenetic development of megaspore mother cells artificially by physical or chemical means. We have used pollen irradiation in several apple scion varieties, making use again of the antho-

cyanin gene in Baskatong to screen out purple hybrid embryos (James *et al.*, 1985, 1986b). Both haploids and diploid maternal-type embryos can be obtained using levels of irradiation beyond 70 krad. These embryos could be cultured and micropropagated without any intervening dormancy phase. The frequency of normal-looking but immature embryos derived from pollen irradiations of 100 krad and above was about 5/1000 flowers pollinated. Some of these embryos could be grown into green-shooted micropropagated cultures, providing a constant source of replicated material for isoenzyme analyses (Menhinick and James, 1986). Preliminary data suggest that these embryos had a sporophytic origin. Apple embryos have a pronounced regenerative capacity (Kouider *et al.*, 1984a) and thus may be of value in the initiation of embryogenic callus for protoplast work. Several of the plants of apomictic origin are currently being tested in field trials to assess vigour and fruit characters.

REGENERATION FROM EMBRYONIC OR SEEDLING TISSUES AND THE VALUE OF EMBRYO CULTURE

Regeneration of plantlets from embryo or seedling tissues for the genetic improvement of existing cultivars of an asexually propagated species has little value except where it provides basic information about the underlying physiological and biochemical mechanisms that control the process of regeneration itself. In this respect the work of Kouider *et al.* (1984a, 1985) on apple and Browning *et al.* (1987) on pear has been valuable in showing the influence of polarity on shoot regeneration from cotyledons. The scale of regeneration from such tissues also emphasizes differences in the ability of juvenile and mature tissues to regenerate and should thus assist investigation of the mechanisms of regeneration.

Production *in vitro* of regenerated and cloned plants from embryonic material could be of great interest. The availability of maternal embryos by induced apomixis could allow the development of an alternative propagation system, either by organogenesis or by somatic embryogenesis. Strategies for genetic manipulation using mature tissues could then be applied to these types of embryos as well. Secondly, the clonal propagation of true-to-type F_1 hybrid embryos derived from dihaploid parents will be essential once haploid production is to be used to full advantage (*see* 'Haploidy', pp. 52–55).

The practice of embryo culture in temperate fruit trees has a long history and has been reviewed recently by Ramming (1983). The high regenerative capacity of embryonic material from several temperate fruit tree species (Liu, Sink and Dennis, 1983; Rubos and Pryke, 1984) provides the opportunity to exploit gametoclonal variation from sexually recombined material. In fruit trees, such as peaches, with a limited genetic base, the exploitation of this aspect of tissue culture is particularly promising. Initial work aimed at regenerating peach plants from immature embryos (Hammerschlag and Bauchan, 1983; Hammerschlag, Bauchan and Scorza, 1985) has recently been used by Hammerschlag (1986) to screen for variants that have increased *in vitro* resistance to *Xanthomonas campestris*, the causal agent of peach leaf spot.

Lack of understanding of the basic mechanisms controlling the *in vitro* germination of immature embryos at present restricts progress in this field.

In vitro mutagenesis

This type of induced variation is best discussed against the background of previous work on mutation breeding carried out *ex vitro*.

Lacey and Campbell (1982) have noted, that temperate fruit trees generally—apples in particular—have great promise for mutation breeding programmes because of their highly heterozygous nature. Induced mutant phenotypes are easy to detect and, as they are asexually propagated, they can be multiplied quickly. Furthermore, many important cultivars of apple are natural sports, produced by spontaneous mutation, often replacing the original selection (Lapins, 1983). This must add weight to the case for mutation breeding (breeding by mutation) as a sensible procedure to be adopted for the genetic improvement of all asexually propagated fruit trees.

Lacey and Campbell (1982) have summarized the results of their apple mutation programme which began at the Long Ashton Research Station in 1970. They listed nine character defects that they believed could be alleviated by mutation breeding in nine cultivars. Only two cultivars, Bramley's Seedling and Cox's Orange Pippin, were assessed in any detail, and these for compact growth and self-fertility. In both cultivars the desired mutant clones with these characters were obtained, although with Bramley's Seedling most of the mutants produced were inferior to the original cultivar and a large number of chimaeras were found (Campbell and Sparks, 1986); some of these were stable, however. In Cox's Orange Pippin the desired self-fertility was achieved in four clones (Sparks and Campbell, 1986), although this has led to higher fruit set than controls and consequently to too many small fruit. Appropriate cultural practice should be able to offset this fault, but from the genetic viewpoint it is important to notice that the appearance, flavour and storage quality of the fruit was not altered in the final selections.

An interesting adjunct to this work was the combined use of micropropagation and *in vitro* chemical mutagenesis. Webster, Sparks and Belcher (1986) have recently reported on the effects of ethane methyl sulphonate (EMS) applied initially to axillary shoots of micropropagated Cox's Orange Pippin. Budwood from these and from untreated micropropagated controls was produced in 1981 and the young trees were grown for 5 years and growth monitored. For the micropropagated controls, these workers reported findings similar to those of Webster *et al.* (1985), in that the trees displayed greater vigour and were slower to flower than conventionally raised trees. The EMS-induced mutant clones displayed a variable response in that both enhanced and reduced vigour of the scion were recorded.

The Long Ashton programme (Lacey and Campbell, 1982) illustrates that it is possible to obtain desired variants of existing commercial varieties using mutation breeding techniques, but that many years of testing and screening are involved and that large populations of plants must be available at the outset. In the case of the cultivar Cox's Orange Pippin alone, over 7000

trees from their irradiated populations had to undergo trials and be assessed on several different sites over several seasons.

In view of the resources and time that need to be devoted to this kind of work, it is perhaps not surprising that there are no other reports of similar detail from other temperate fruit trees, although Lapins (1974) has successfully introduced self-fertility and compactness into the cherry cultivar Stella.

COUPLING *IN VITRO* MUTAGENESIS WITH SOMACLONAL VARIATION

The question that now has to be asked is 'Can any of the techniques of somaclonal variation and/or *in vitro* mutant induction and regeneration improve on these results?'

Irradiating whole buds and then propagating subsequently from such material will often lead to the formation of chimaeral tissues, as not all histogenic layers will be equally affected by the irradiation treatment. Over the past century, natural and induced mutations have led to several types of chimaera (periclinal, mericlinal, etc.) in apple, pear, sweet cherry and peach (Pratt, 1983). The arrival of cell and tissue culture techniques for these species means that a greater amount of plant material can be mutagenized per unit time and per unit space, because buds are usually much smaller than their *in vivo* counterparts. However, the techniques do not reduce the possibility of chimaera formation.

It was realized many years ago that generating adventitious buds from a known chimaera was a satisfactory way of obtaining homohistont* plants (e.g. Dermen, 1948, 1955) and Campbell and Sparks (1986) showed reversion of their compact apple mutants to the vigorous parent genotype in shoots regenerated from root cuttings, i.e. the L3 layer. According to Broertjes and Van Harten (1978), coupling *in vitro* adventitious bud techniques with mutagenesis is a much superior way of generating non-chimaeral mutants. They claim that since adventitious bud regeneration is much easier to induce in *in vitro* systems, and mutations arise in single cells, tissue culture systems are ideal for producing 'solid'† mutants. Where the crop is vegetatively propagated, then in theory such mutants may be readily cloned by micropropagation. This would be true if all adventitious buds (or somatic embryos) always originated from single cells, a claim that does not find universal support (*see* for example, Williams and Maheswaran, 1986).

Adventitious bud regeneration techniques are now available (*Table 3*) and it is possible, in one apple variety at least, to generate 150–200 adventitious buds in a volume the equivalent of a Petri dish. Leaf pieces have also been X-irradiated before regeneration of adventitious buds (James, Passey and Rugini, 1987) and the buds cloned by micropropagation. At East Malling we have produced unirradiated (R1 plants) and irradiated (M1R1 plants)

*Plants regenerated from one histogenic layer and therefore non-chimaeral.

†Mutant obtained where all component cells carry the mutation, i.e. a non-sectoral chimaera.

regenerated apple shoots of the cultivar Greensleeves for field trials in a comparison of somaclonal variation with X-ray-induced mutagenesis. Initial plants regenerated after irradiation are not distinguishable from unirradiated controls and we have seen no evidence of chlorophyllous mutants or gross changes in phenotype. Douglas (1986), working with a tree species (*Populus* sp.), also could find no signs of aberration in the plants he regenerated from callus, either with or without irradiation.

In conclusion, the contribution of *in vitro* mutagenesis, coupled with adventitious bud techniques, to genetic improvement in fruit trees is still unknown and the spectrum of change induced, compared with somaclonal variation, will be answered only after the trees have cropped within the next 5 years.

CONDITIONS AND CONSIDERATIONS WHEN USING *IN VITRO* MUTAGENESIS

Assuming that *in vitro* mutagenesis and adventitious bud techniques are available, or will become so, in all temperate fruit trees, under what conditions should the methods be used? The work *in vivo* has shown that it is possible to reach the desired goals even when little is known about the genes that control the characters in question. The methods are likely to be most effective when the character desired is controlled by a single gene, and where this is expressed only in the homozygous recessive condition, as most mutations are from the dominant to the recessive form of the allele. Unfortunately, this condition is rarely found in fruit trees, where most of the economically important traits, e.g. vigour, yield, growth type, precocity, fruit characters, are polygenically controlled. In addition, the high degree of heterozygosity means that many homozygous recessives may be induced, most of which would be expected to be deleterious.

Nevertheless, favourable gene combinations may be expected in low frequency, but these have to be screened from large populations. *In vitro* methodology could help here in reducing the number of chimaeras and in producing a large number of mutants in a small space. In a cultivar giving 90% regeneration from leaf discs with five shoots per disc, more than 100 shoots can be produced per multi-welled dish. We have estimated that one person could propagate 1000 adventitious buds per day quite easily. Assuming an induced mutation rate of approximately 1%, about 10 mutants per day could be produced. As the regenerants might also be expected to exhibit some degree of somaclonal variation as well, the frequency may well be higher. However, unless there is some way of screening very early for the trait in question, all shoots have to be grown on and tested, a situation that is similar to that faced by the Long Ashton apple breeders nearly 20 years ago. Only if the mode of action for the gene in question is known can one attempt to screen such a large population *in vitro*. This emphasizes the need to characterize the induced mutants by biochemical, physiological, immunological or molecular screening. This, of course, is possible only if the mutations can be expressed and quantitated at the *in vitro* stage and

then correlated with field performance. A good example would be the induction of dwarf mutants where the development of *in vitro* grafting procedures could be used to screen large numbers of mutant, potentially dwarfing rootstocks. Other tests need to be devised for other important traits such as disease resistance, drought and frost tolerance, ease of propagation, etc. Progress in devising such tests is usually hampered by lack of fundamental knowledge of the mechanisms that determine resistance, tolerance, dwarfingness, etc.

Pratt (1983) has reviewed the parameters that should be considered when using conventional mutation breeding procedures and points out that the use of chemical mutagenesis has been hampered by the general inaccessibility and awkwardness of mutagenizing greenhouse or field-grown tree fruit buds. A great advantage of an *in vitro* approach is that, if chemical mutagens are used, the penetration of these into tissues will be much more efficient because the target tissues can be exposed to the mutagen under controlled conditions of dose, temperature, pH, etc. Furthermore, the ability to regenerate from unorganized tissues such as leaf discs minimizes the chimaeral problem. These advantages over conventional mutation methods could well see a revival of interest in mutagenesis *in vitro* for fruit tree breeding programmes.

Although irradiation doses and LD_{50}s are known for practically all conventionally grown material of temperate fruit trees, there is a dearth of such information for tissues *in vitro*. *Table 4* shows the LD_{50}s for a range of tissues from several fruit species using X-irradiation. The LD_{50} for tissue cultured material is lower than that from dormant bud material. The different physiological state and differences in conditions and dose rate may account for this. For instance, in many asexually propagated crops, including fruit trees, actively growing buds have produced more mutants after irradiation than dormant ones (Lapins, 1969; Broertjes and van Harten, 1978). This is presumably due to the fact that cells in the 'S' phase of division are more likely to accumulate point mutations as DNA replication is proceeding, than are dormant cells which are undergoing little, if any, nucleic acid synthesis

Table 4. LD_{50}s and X-irradiation in different temperate fruit trees

Fruit	Plant material	LD_{50} (krad)	Dose* (rad/min)	Reference
Apple	Dormant buds	6·1	1500	Lapins, 1965
	Shoot cultures	2·5	500	James, Passey and Baker, 1986
	Leaf disc regeneration	1·4	500	James, Passey and Rugini, 1987
Apricot	Dormant buds	4·0	185	Donini, 1975
Cherry	Dormant buds	4·0	185	Donini, 1975
	PMC	0·8	110	Lewis and Crowe, 1954
Peach	Dormant buds	3·5	10–53	Donini, 1975

* X-rays used in all cases
PMC = pollen mother cell

and will be in the G_0 phase. A certain amount of control over the cell cycle is possible in cultured tissues, in that most leaf cells, for instance, are differentiated and held at the G_0 stage. Leaf pieces can be irradiated at this stage and potential mutants regenerated or, alternatively, a preliminary growth period can be induced using hormones to start cell division. Mutagenesis can then be carried out before organ formation occurs to prevent chimaera formation. One might expect a different spectrum of variation, depending on the method used, but this is best determined using a dominant visual marker gene.

SUITABLE GENES FOR *IN VITRO* MUTAGENESIS

The anthocyanin gene, *R*, is available in apple as an aid to efficient application of mutagenesis to fruit tissues *in vitro*. By producing hybrid embryos that are all heterozygous red, *Rr*, for anthocyanin formation and then obtaining micropropagating shoot cultures, a vast amount of genetically and visually marked leaf material is available. The leaf tissues can be mutagenized and shoots regenerated from them. Mutants that are rendered homozygous recessive for the anthocyanin gene (*rr*) will be green whereas those unaffected and arising via diplontic selection will be heterozygous red (*Rr*). In this way the relative efficiencies of different methods of physical and chemical mutagenesis and the effects of different dose rates could be quantified entirely *in vitro* without producing a single tree. Such information would be invaluable in formulating precise mutagenic regimes for the most efficient production of colour mutants in other commercial cultivars assuming that, as in both apple and pear, colour mutations are carried in the L_1 layer (Pratt, 1983).

Another dominant gene of interest in this connection is the *Co* gene (Lapins and Watkins, 1973) that controls the compact, dwarf habit in apple and is evident in the two mutants McIntosh Wijcik and McIntosh Bendig (*Figure 3*). Seedling selections from McIntosh Wijcik crosses performed at East Malling (Tobutt, 1984) also carry the gene as a dominant and are efficiently regenerated from leaf discs as adventitious buds in culture (James, Passey and Rugini, 1987). In this case, reversion to the homozygous recessive condition should result in the loss of the compact habit. As it is claimed that the gene can be characterized *in vitro* on the basis of its cytokinin tolerance (Lane and Looney, 1982) it should be possible to screen for mutations at the *in vitro* stage. The possible value of this gene to future genetic transformation work will be mentioned later. Similar dominant genes exist in the pear cultivar Nain Vair (Decourtye, 1967) and in peach (Monet and Salesses, 1975) although in two different cherry cultivars the dwarf compact trait was not transmitted to the progeny (Lapins, 1976).

The successful production of economically useful fruit tree mutants using *in vivo* methods of mutagenesis should now form a basis for mutation breeding programmes based on *in vitro* procedures and adventitious bud techniques. Once regeneration protocols have been developed, the way is open for the release of a whole new spectrum of variation and for the production of valuable new germplasm.

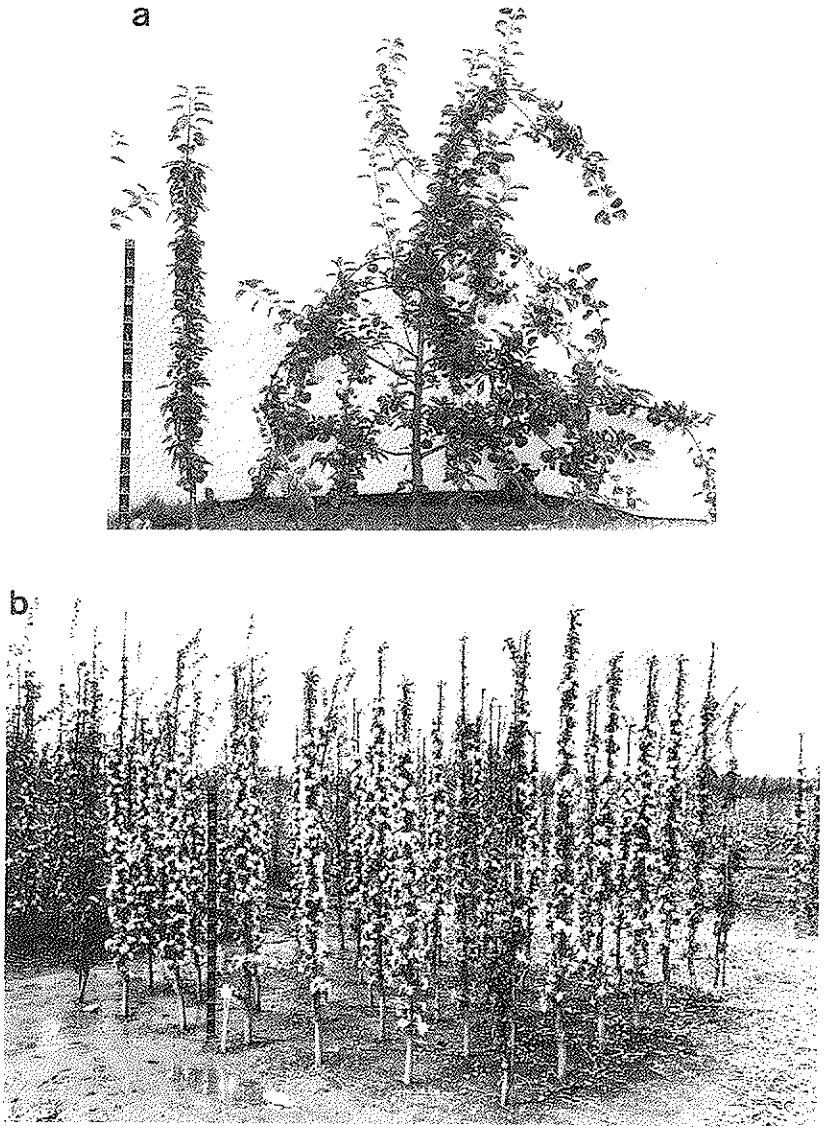

Figure 3. (a) The effect of the single dominant gene '*Co*' on apple phenotype: left 'Wijcik' on the rootstock MM106, right, normal phenotype in parental line McIntosh. Both trees 3·5 years after planting. (b) 'Wijcik' trees in full bloom, 4 years after planting.

Ploidy manipulations

The genetic, biological and agronomic significance of ploidy changes in crop and non-crop species has been dealt with in some detail elsewhere (Lewis, 1980). In addition, Sanford (1983) has admirably reviewed the process as it relates to all fruit species and gives many examples of where and how the process has been used in temperate fruit tree breeding.

The purpose of this section is to review ways in which tissue culture methodology can improve upon more established protocols for manipulating the ploidy level of temperate fruit trees.

The three types of ploidy discussed here are:

1. Haploidy;
2. Triploidy;
3. Ploidy doubling.

HAPLOIDY

Haploidy is relatively rare among temperate fruit tree species and, even where it has occurred, it has had only a minor role in fruit tree breeding. This is surprising because pure breeding lines from dihaploids in a tree species would be invaluable for F_1 hybrid seed production and for uncovering useful recessive genes that can later be used in breeding programmes. Radojevic and Kovoor (1986) have extensively reviewed the specific benefits that haploids have in tree breeding, including the induction of self-compatibility, a character of particular interest in fruit trees. In this review the value of the anthocyanin gene *R* in the homozygous condition in apple has already been discussed (page 50).

Haploids can arise from microspore mother cells of the pollen or from megaspore mother cells of the ovule. In most species anther culture has typically been used and methods for producing haploids by this technique are the subject of numerous tissue culture reviews (*see*, for example, Sunderland, 1983). Producing haploids via unfertilized and fertilized ovules has been less studied, but has been important in many species, notably in barley where haploids may efficiently be produced using the so-called 'bulbosum' technique (Kasha and Kao, 1970).

There are only two reports, both from China, of viable haploid plants of temperate fruit trees being produced through anther culture and these both in apple (Fei and Xue, 1981; Wu, 1981). Radojevic and Kovoor (1986) list the details for anther culture of cherry and pear (Jordan, 1975) and apple (Kubicki, Telezynska and Milewska-Pawliczuk, 1975; Milewska-Pawliczuk and Kubicki, 1977). No viable plants were produced from any of this work although proembryos, callus and leafy callus could be produced in different cases. James and Wakerell (1982) also cultured anthers of several apple and cherry cultivars on various media, with a range of pretreatments and at different stages of flower development. Like so many other tree species, only callus was formed.

Temperate fruit trees pose special problems for anther culture. Seasons restrict sampling times to a few days a year, unlike herbaceous crops or even subtropical fruit trees, the flowers of which can be made available at almost any time. Temperate fruit tree flowers, however, demand continuous use of controlled-environment cabinets and a large supply of cold-stored (−1°C) potted trees. These resources are often not available.

In a dedicated breeding programme at Angers, France, haploid apple plants have been successfully produced by Lespinasse, Godicheau and Duron

(1983) without recourse to tissue culture. They selected green seedlings after hand pollination using the same homozygous marker gene *R* for anthocyanin but carried by *Malus p. niedzwetzkyana* rather than Baskatong. They also produced the dihaploid after taking haploid vegetative tips into culture and doubling with colchicine. Lespinasse's group report the production of five different haploids from four different varieties, Topred Delicious, Erovan, Querina, and Golden Delicious. Of these, three are genetically similar, Erovan and Topred Delicious being obtained by mutation from Delicious, indicating that haploid production is variety dependent as no haploids were obtained in the variety Liberty although 4000 seedlings were examined. The proportion of haploids obtained was about 1/1000 seedlings which represents about 1/100 flowers in apple assuming 10 seeds per fruit and 100% fruit set. All their haploids were characterized by the same phenotype—a slender stem and thin small narrow leaves.

Recently, as part of our programme to produce maternal diploids in apple via pollen irradiation using the pollen marker gene in Baskatong, we have fortuitously produced a haploid in the variety Greensleeves. This variety is a Golden Delicious × James Grieve cross. The haploid was cultured as an immature embryo and micropropagated for 2 years as green shoot cultures before being confirmed as a haploid from chromosome counts of shoot tips (Menhinick and James, 1986). Its morphology in culture was distinctive in that the shoots were slender and the leaves narrow and about half the size of the corresponding diploid (*Figure 4*).

The Greensleeves haploid multiplies quickly in culture and many thousands of shoots have been produced. However, it has not yet been induced to root *in vitro* although the diploid parent gives 100% rooting using the same rooting protocol.

The frequency of haploid production using pollen irradiation was about 1/100 flowers pollinated. Assuming 10 seeds per fruit, this represents one haploid per 1000 pollination events, which is the same ratio of haploid seedlings obtained by Lespinasse and co-workers (Lespinasse *et al.*, 1983) although they did not use pollen irradiation. The mortality of cultured immature embryos from 75 krad pollen irradiation (gamma source) was high and, although many of them looked normal at first, they failed to grow on normal micropropagation media (*Table 5*). Pollen irradiation doses from 0·5 to 150 krad have been used and haploid plants have only ever been produced after doses of 70–75 krad. Below this dosage aneuploids may be obtained and from 100 krad and above the rare embryos that are found develop into normal-looking shoot cultures (Menhinick and James, 1986). Raquin (1986), working with *Petunia* ovary culture and pollen irradiation, found haploids after pollination with pollen treated with 70 krad. This apparent specificity of dose is at variance with the recent finding that apple haploids can be produced from doses as low as 10 krad (Y. Lespinasse, personal communication). Our own findings indicate that all embryos derived from crosses using this level of irradiation carried the anthocyanin marker gene and therefore were euploid or aneuploid hybrids.

From these figures it seems that apple haploids may be produced at a

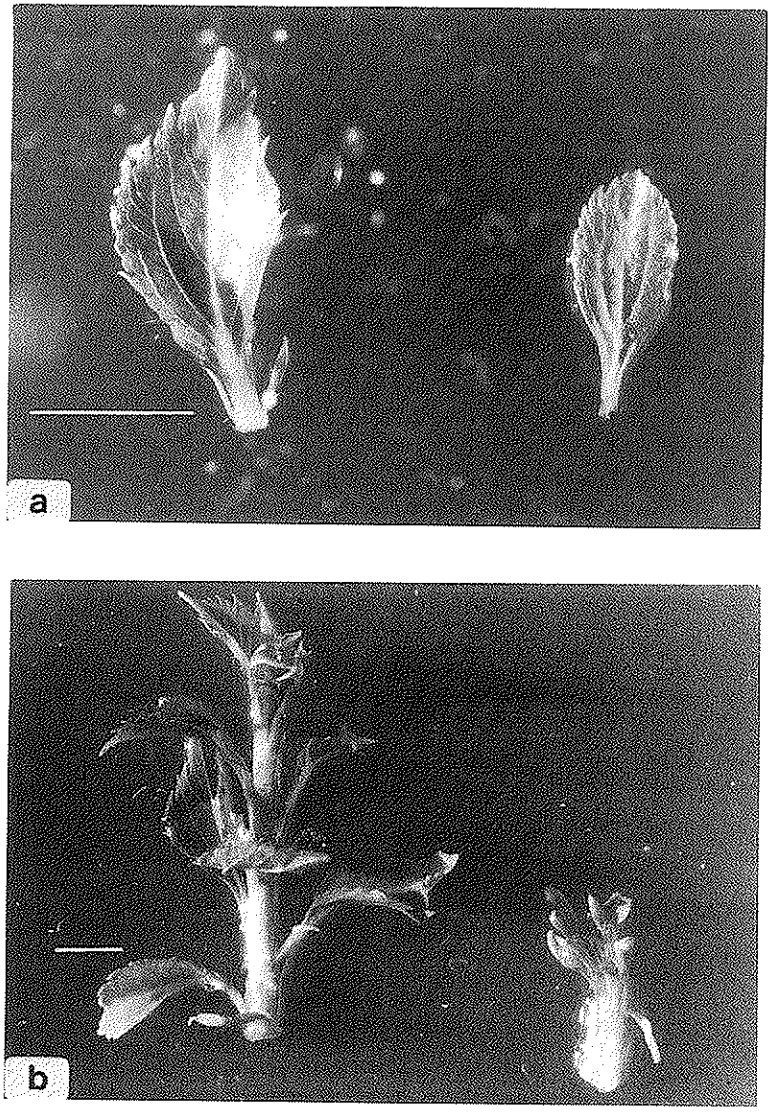

Figure 4. Diploid and haploid phenotypes of the apple cultivar Greensleeves. (a) Typical leaf sizes from unrooted shoot cultures: left, diploid; right, haploid. (b) Typical single shoots from proliferating shoot cultures: left, diploid; right, haploid. Bar = 0·5 cm.

frequency of about 0·1%. In a ten-seeded fruit this means that about 100 flowers need to be pollinated, a task taking 2–3 h. If the conditions that promote embryo development in culture can be improved so that micropropagating cultures are obtained, this seems a much more efficient method for temperate fruit trees than anther culture.

Haploids also arise naturally in peach populations, as reported by Hesse (1971) and Toyama (1974); Hesse reported a frequency of 0·1%, the same

Table 5. Frequency of haploid production in different apple cultivars after pollination with Baskatong pollen irradiated at 75 krad gamma irradiation (1985 season)

Cultivar	Number of flowers pollinated	Number embryos found	Embryos/ flower pollinated	(%)	Embryos becoming green shoots	Haploids
Idared	400	9	1/44	(2·2)	0*	—
Greensleeves	107	1	1/107	(0·9)	1	1
Spartan	416	5	1/123	(0·8)	0*	—
Queen Cox	212	3	1/70	(1·4)	1	0

* Cultured embryos failed to survive

as that for apple. There are no other reports of haploidy, either natural or induced, in temperate fruit trees.

TRIPLOIDY

There are three ways of producing triploids: (1) endosperm culture; (2) inducing the formation of the tetraploid by sexual or somatic doubling of chromosome number and then crossing with a diploid, and (3) protoplast fusions of diploid and haploid cells. This last method is discussed under 'Protoplast Technology'.

Endosperm culture

The biological relevance and applications of endosperm culture to all crops have been reviewed by Johri, Srivastava and Raste (1980). The double fertilization process in angiosperms ensures that endosperm tissue is initially triploid. However, in most temperate fruit trees it is an ephemeral tissue (non-endospermous seeds) which means that, as in the case of the nucellus, the researcher has only a limited time in which to sample seeds and culture the tissue. Although the endosperm has been much used in physiological and biochemical studies, there have been very few attempts to produce triploid plants from this tissue.

The production of triploids by endosperm culture has two major implications for fruit trees: (1) triploid fruit are often larger than their diploid counterparts, and (2) because of their lowered fertility the fruits have few, if any, seeds. Depending on the fruit in question, one or both or neither of these characters may be important. In apple for instance many important cultivars are triploid, but their lowered fertility does not unduly interfere with fruit set (Brown, 1975). In pears, on the other hand, this is not so where generational sterility leads to reduced seed set (Layne and Quamme, 1975).

There are already reports of triploid plants being regenerated from endosperm callus of tree species such as *Citrus* (Wang and Chang, 1978) and sandalwood (Lakshmi Sita, Raghava Ram and Vaidyanathan, 1980). Mu and

Liu (1979) cultured apple endosperm using both an induction medium (BA, 2,4-D) and a differentiation medium (BA, NAA). The callus produced contained only 2–3% triploid cells, the remainder having chromosome numbers varying from 17 to more than 200. Nicoll, Chapman and James (1987) have recently confirmed these wide ploidy ranges using Feulgen densitometry. James, Passey and Deeming (1984) cultured apple endosperm from eight cultivars using immature seeds 50 days post-pollination. They showed that the extent of callus growth was strictly cultivar dependent and that callus formation occurred in both the presence and absence of exogenous hormones. Hormone-autotrophic callus had fresh weight doubling times of 10–20 days, depending on the cultivar, making it extremely useful for protoplast studies (James *et al.*, 1986a). No differentiation of shoots or roots was ever seen in any of these cultivars. In contrast Mu *et al.* (1977) were able to obtain shoots from endosperm callus using the growth regulators BA and NAA; in their case, however, the triploid nature of the plants was not established. In peaches, too, the endosperm has been used to form first callus and then plantlets (Meng and Zhou, 1981).

So far there have been no confirmed reports of triploid plants being produced from the endosperm of temperate fruit trees, although there is no reason why this should not be achieved. It would clearly be much quicker than the alternative strategy of producing an autotetraploid and crossing with a diploid. The endosperm is a dynamic tissue and during its short life in non-endospermous seeds is nutritionally dependent on the intense meristematic activity of the peripheral layers of cells adjacent to the nucellus. This meristem is continuously cutting off cells that enlarge, differentiate and move towards the embryo interface where they break down to supply nutrients to the developing embryo. The culture of the meristematic layers rather than the whole endosperm might reduce the frequency of non-triploid cells and callus growth.

It would be well to bear in mind that any triploid plants produced by endosperm culture will only be a 'type' of the initial cultivar in question because all three stem nuclei are haploid and the products of genetic recombination. Consequently they must be treated as seedling progeny. The possibilities of obtaining diploid homozygous endosperm tissues in apple after pollen irradiation have been discussed by Nicoll and co-workers (Nicoll, Chapman and James, 1987).

PLOIDY DOUBLING

Allo- and autopolyploids of several temperate fruit trees have been in existence for some time and their derivation, uses and limitations have already been comprehensively reviewed by Sanford (1983). The production of autopolyploids by somatic doubling using agents such as colchicine has been practised for some time in fruit trees (e.g. Lapins, 1975). The advent of tissue culture regeneration techniques for temperate fruit trees should see a marked improvement in the efficiency of production of 'solid' non-chimaeral polyploid mutants. The ability to initiate and control adventitious shoot

formation *de novo* from somatic cells means that the doubling agent can be used to generate polyploidized meristems that subsequently produce homohistont plants. Moreover the *in vitro* approach ensures more efficient and controlled contact between colchicine and its target cells.

In embarking on any ploidy doubling procedure (regardless of whether tissue culture techniques are used), the importance of retaining heterozygosity should be considered. Sanford (1983) sees the greatest use of artificially doubled polyploids for making new cultivars by interploid crossing. Apples and pears are good examples, where the production of triploids is possible by crossing tetraploids with diploids. These triploids then profit from polyploid heterosis. In pears tetraploids would be valuable in that they would show greater self compatibility.

James, MacKenzie and Malhotra (1987) have recently modified an adventitious bud technique using root callus (Druart, 1980) to produce several clones of hexaploid cherry rootstocks from the sterile triploid, Colt ($2n = 3x = 24$). Colt is of considerable commercial importance and incorporates a number of highly desirable horticultural features (Pennel *et al.*, 1983); however, it is only semi-dwarfing as a rootstock. The aim therefore was primarily to introduce fertility to ensure its entry into breeding programmes and to assess its capacity to control scion size. As a callusing phase is also involved during the shoot regeneration phase, this experiment also afforded us an opportunity to assess the extent to which variants could be produced by the process of somaclonal variation.

The technique consisted of feeding colchicine to the base of single micropropagated shoots simultaneously with auxin in an attempt to produce hexaploid root primordia. After a few days the individual shoots were transferred to a root growth medium lacking colchicine and auxin and the putative hexaploid roots were allowed to emerge over a period of a few weeks. They were then transferred to a third medium to regenerate shoots and embryoid-like structures from the roots. A variation of this technique was tried in which colchicine was not present during root primordium formation but was present in the root growth medium. Triploid root primordia would therefore grow out in the presence of colchicine. Regeneration then took place in the absence of colchicine. A considerable number of regenerated plants were then micropropagated, rooted *in vitro*, transferred to soil culture and have now entered field trials.

Chromosome counts of root tips from regenerated plants showed that both methods produced hexaploid plants but that about 70% of clones that had been subjected to colchicine were still triploid. Such non-polyploids, however, may well be variants of the original triploid, as colchicine has produced changed phenotypes without altering ploidy level in sorghum (Franzke and Ross, 1952), barley (Gilbert and Patterson, 1965) and *Solanum* (Hermsen, Wagenvoort and Romanna, 1970).

Hexaploid cherry plants were distinguishable from triploids both at the *in vitro* and *in vivo* stages of growth on the basis of leaf morphology and degree of 'greenness'. In all, 24 clones were produced, of which nine were hexaploid, 14 were still triploid and one was a hexaploid/triploid chimaera as judged

by root tip squashes. The chimaera had the phenotype of the hexaploid. Measurements of stomatal size and density correlated extremely well with differences in ploidy, the hexaploids having 50% longer stomata and a correspondingly reduced density compared with all triploids. Plants taken to the greenhouse stage have shown no evidence of somaclonal variation within the two ploidy classes for vegetative characters such as leaf morphology and stomatal density.

In apple rootstocks, Beakbane and Majumder (1975) showed that the lower the stomatal density the more dwarfing the effects of the rootstocks on the grafted scion. Stomatal density is usually inversely related to guard cell size and this may then be related to other factors that directly control the vigour of the scion. If stomatal density can truly be shown to be correlated with vigour in any regenerated rootstock material, there is real hope that *in vitro* screening for this major horticultural character would be possible.

Protoplast technology

The use of protoplasts for genetic manipulation work can be considered under two headings:

1. Protoplast culture and regeneration to plants;
2. Somatic hybridization.

PROTOPLAST CULTURE AND REGENERATION TO PLANTS

Until recently the use of protoplasts was considered essential for genetic engineering work where the aim was to transfer foreign genes into the crop of interest. This presented possibilities of transforming directly with vectors, or even by encapsulating naked DNA in liposomes (*see* review by Gunn and Day, 1986). Views changed somewhat when Horsch *et al.* (1985) showed that it was possible to obtain transformed plants without recourse to protoplast technology. They co-cultured tomato leaf discs with 'disarmed' vectors of *Agrobacterium tumefaciens* and obtained transformed regenerated plants. This opened up the possibility of transforming any plants that could be regenerated from complex explants.

It has also been found that considerable somaclonal variation could be obtained in plants regenerated from complex explants without recourse to regeneration from protoplasts. Nevertheless, protoplasts offer unique opportunities for genetic improvement in temperate fruit trees, particularly as trees are normally composed of a rootstock and scion. The ability to fuse protoplasts makes possible the production of novel somatic hybrids from rootstock/scion fusions and the synthesis of new fruit species. However, although numerous species can now be regenerated from protoplasts (*see* e.g. Pental and Cocking, 1985), there is at the time of writing only a single report of plant regeneration from protoplasts of a temperate fruit tree subject and this in wild pear, *Pyrus communis* var. *Pyraster* L., (Ochatt and Caso, 1986).

Kouider *et al.* (1984b), working with the apple cultivar Jonathan, were able to obtain embryo-like structures from protoplast-derived callus and these could be induced to form roots but no complete plants were obtained. Hurwitz and Agrios (1984) were also unable to regenerate plants from apple protoplasts derived from cell suspensions. James *et al.* (1986a) obtained micro-calli from apple endosperm protoplasts, but these ceased to grow after several weeks in culture. Matsuta *et al.* (1986) also obtained callus colonies from peach (*Prunus persica* L. Batsch, cv. Hakuho) protoplasts isolated from cell suspensions originally obtained from leaf callus. The protoplast-derived callus did not regenerate plants. All these groups of workers used fast-growing cell suspension cultures as the source material; this was, itself, material which had no capacity for regeneration.

Work using apple leaf mesophyll protoplasts has also been disappointing. Kouider and co-workers (Kouider *et al.*, 1984b) and James and co-workers (James *et al.*, 1986a) obtained zero or negligible levels of protoplast division in digested leaves obtained from *in vitro* cultures. The importance of reducing agents such as 2-mercaptoethanol and the sulphur-containing amino acid L-methionine in increasing the yield of apple leaf protoplasts has been demonstrated by Wallin and Welander (1985).

Ochatt and Caso (1986) regenerated several pear plants from mesophyll protoplasts but their success was cultivar dependent, several other pear genotypes being recalcitrant (S. G. Ochatt, personal communication). The plant growth regulators that eventually caused regeneration—BA (1 mg/ml) and IBA (0·1 mg/l)—were used in the same concentrations that have given regeneration from embryoid-like structures from apple leaves (James, Passey and Malhotra, 1984) and that have been used for some years to micropropagate apples and pears (Jones, 1976).

The wild pear plants produced by Ochatt and Caso (1986) after protoplast culture showed a range of phenotypes with regard to leaf morphology and rooting ability. An abnormal leaf phenotype was correlated with inability to root and abnormal chromosome number, whereas those with a more normal leaf morphology rooted well and had the euploid chromosome number (Ochatt, 1986). More recent work has seen the successful production of shoot-like structures from protoplast-derived callus of the cherry rootstock Colt (S. G. Ochatt, personal communication).

It is to be hoped that these initial successes will be followed by others so that successive somatic hybrid strategies may eventually be implemented.

SOMATIC HYBRIDIZATION

The many potential applications and uses of somatic hybrid plants to plant breeding have been discussed in detail (Cocking, 1983; Shepard *et al.*, 1983). The fusion of protoplasts by chemical or physical methods is a relatively easy procedure and has already been demonstrated in apple (James *et al.*, 1986a). It is the recovery of the somatic hybrid plants from these heterokaryons that often poses problems. Where regeneration protocols are not even available

for either of the parental lines (as is the case for practically all fruit trees) the production of true hybrids does seem a remote prospect. Nevertheless the extension of successful protoplast fusion techniques to fruit trees would have major impacts on propagation, breeding and fruit production.

There are many possible 'somatic hybrid targets' to aim for in temperate fruit trees. These would include both rootstock/scion fusions and new hybrid rootstocks. New rootstocks are a particularly good target because fertility in an asexually propagated rootstock is of little or no consequence. Some examples are:

1. Cherry–plum fusions for the transfer of plum pox resistance to the hybrid rootstocks, as all cherries are resistant to the plum pox virus. A good example would be 'Pixy' (plum, *Prunus insititia*) fused with Colt (*P. pseudocerasus*) to produce a 9x *Prunus* combining plum pox resistance from cherry with the more dwarfing habit of plum;
2. Pear–quince (*Cydonia*) fusions for the production of rootstocks that have greater dwarfing capacity and better scion compatibility than existing stocks;
3. Apple–pear rootstock fusions for the introduction of better dwarfing and greater compatibility with pear scions;
4. Fusion of haploid *Malus* protoplasts carrying scab and mildew resistance with a diploid commercial variety to produce disease-resistant triploids.

It now appears that protoplast fusion techniques will be most useful for transferring a limited amount of genetic material from an alien species into the crop species of choice (Cocking, 1986; Gunn and Day, 1986). Such fusions also present novel ways of studying the recombination and reassortment of cytoplasmic and mitochondrial genomes and their contributions to the phenotype. In fruit trees, organellar genetics is a completely unexplored field so that the importance of cytoplasmic inheritance in determining phenotype is unknown. Somatic hybrids could make a considerable contribution to an understanding of such basic phenomena as the 'rootstock effect'. The rapid advance of molecular genetics and the synthesis of marker genes means that in the next few years these should be available for hybrid cell selection procedures.

The importance of triploids in several fruit tree species has been discussed earlier. A novel way of producing triploids is discussed by Pental and Cocking (1985) and involves fusing haploid protoplasts from pollen tetrads with diploid protoplasts of the desired cultivar. In fruit trees that are very floriferous and multi-ovuled, reduced fertility is less of a consideration than in seed-propagated species and often minimal 'set' is required for fruit growth. Several wild species carry disease resistance and would make suitable donors of haploid protoplasts.

Genetic transformation using Agrobacteria

There are now many publications dealing with transformation of dicotyledonous plant cells and tissues using both wild type and genetically engineered

strains of the common soil bacterium *Agrobacterium*. The whole subject of *Agrobacterium*-mediated transformation has also recently been reviewed (e.g. Bevan and Chilton, 1982; Hooykaas and Schilperoort, 1984; Nester *et al.*, 1984). Current strategies for gene transfer in these plants rely on the natural ability of *Agrobacterium tumefaciens* and *A. rhizogenes* to transfer specific genes to their host plant via the plasmids they harbour. These are designated tumour-inducing (Ti) in *A. tumefaciens* and root-inducing (Ri) in *A. rhizogenes*. These plasmids possess all the genes for gaining entry into host cells and for either tumour induction (Ti plasmids) or root formation (Ri plasmids) once there. Only a small section of the plasmid DNA, the T-DNA, is incorporated and functional within the host genome (e.g. White *et al.*, 1982; Willmitzer *et al.*, 1982). This T-DNA then codes for all proteins associated with gall or root formation. Some of these proteins are enzymes that manufacture the opine type of amino acid that provides a unique source of carbon and nitrogen usable only by the invading organism.

TISSUE CULTURE TRANSFORMATION

Much of the earlier work on transformation *in vitro* has been carried out using tobacco protoplasts whereby the protoplasts were co-cultured with the bacteria. In these cases efficient regeneration protocols for the protoplasts had been defined (Potrykus *et al.*, 1985). More recently the use of electroporation and plasmid DNA has markedly increased the efficiency of transformation to 1–3% in a range of species (Shillito *et al.*, 1986). When the development of efficient regeneration systems from protoplasts of temperate fruit tree species becomes routine these techniques will, no doubt, be used; however, until then it is likely that the simpler methods of transforming tissues such as leaf or stem explants (Horsch *et al.*, 1985) will be used.

An alternative method to co-culturing has also been devised by Ooms, Karp and Roberts (1983) and Ooms *et al.* (1985) whereby shoots grown *in vitro* are infected using a sterile scalpel carrying an inoculum of the bacteria. With this technique Ooms and co-workers were able to obtain transformed potato plants using wild-type plasmids in *A. tumefaciens* and *A. rhizogenes*. They used the Ti plasmid T37 to produce galls and the Ri plasmid pRi1855 to produce transformed roots. In both cases plants were regenerated from the galls and the roots respectively, although long culture periods were necessary. The regenerated plants had characteristic phenotypes that could be assessed *in vitro*: for example the Ti plants formed masses of small shoots on hormone-free medium and could not be induced to root; consequently, they needed to be grafted on to rootstocks.

Plants have been regenerated from Ri-transformed roots of several dicotyledonous herbaceous species (Ackermann, 1977; Spano and Costantino, 1982; Ooms *et al.*, 1985) and have been reported as having the Ri phenotype (Tepfer, 1983). This phenotype has a number of interesting characteristics which are dealt with below because of their particular relevance to fruit tree improvement.

TRANSFORMATION OF FRUIT TREES *IN VITRO* USING AGROBACTERIA

Temperate fruit trees are ancient hosts to *Agrobacterium* spp. and their susceptibility to *A. tumefaciens* often leads to the development of crown gall disease or of hairy root disease when *A. rhizogenes* is involved (Riker, 1930). Paradoxically, this very susceptibility may in future be turned to advantage by transforming fruit tree tissues *in vitro* using both wild-type and genetically engineered 'disarmed' vectors of Agrobacteria.

Wild-type plasmids

Work with other species has confirmed that transformed plants have altered physiological and biochemical characteristics, including altered endogenous hormone status, which is probably largely responsible for the changed phenotypes observed (Ooms and Lenton, 1985). Fruit tree subjects with these phenotypes are of interest to basic science and possibly to fruit tree agronomists. Altered endogenous cytokinin levels may have important effects on the branching character of scion varieties and on flowering and cropping behaviour. Ooms, Karp and Roberts (1983) have already shown that there were marked effects on the reproductive physiology of Ti-transformed potato plants where basic physiological mechanisms such as fruit set and subsequent abscission were perturbed. These processes in fruit trees are of great importance but poorly understood. The existence of Ti-transformed fruit trees could provide valuable insights into the basic mechanisms that control these processes.

There is scant reference to the use of *in vitro* transformation work on fruit trees, although some of the first reports of the effects of *Agrobacterium* on plant species was described in apple seedlings (Riker, 1930). At East Malling we are using both the shoot injection and co-culturing procedures using leaf pieces. Using the injection method of Ooms and co-workers (Ooms, Karp and Roberts, 1983) several fruit tree subjects have now been assessed for their susceptibility *in vitro* (James, Murphy and Passey, 1985; James, Passey and Marks, 1986; Browning *et al.*, 1987). Stems of several fruit tree cultivars have been inoculated with strains of *A. tumefaciens* harbouring the nopaline-producing plasmid pTi T37, and with *A. rhizogenes* strain LBA 9402 carrying the plasmid pRi1855 and coding for agropine (*Figure 5*). There is considerable variability in response to both strains of Agrobacteria (*Table 6*) : apple cultivars generally form galls rarely, whereas all cherry and plum cultivars have a high level of response, and pears have an intermediate response. There were both intra- and interspecies differences in response to *A. rhizogenes*: apple rootstocks were responsive but the scion varieties were not, while cherry and plum formed roots only occasionally; pears were very responsive.

Although shoots could be regenerated from galls at a low frequency, either spontaneously or by exogenous hormone application, in three of the four genera these either failed to survive cloning or, if they did survive, they gave a negative response for nopaline although excised galls almost always gave

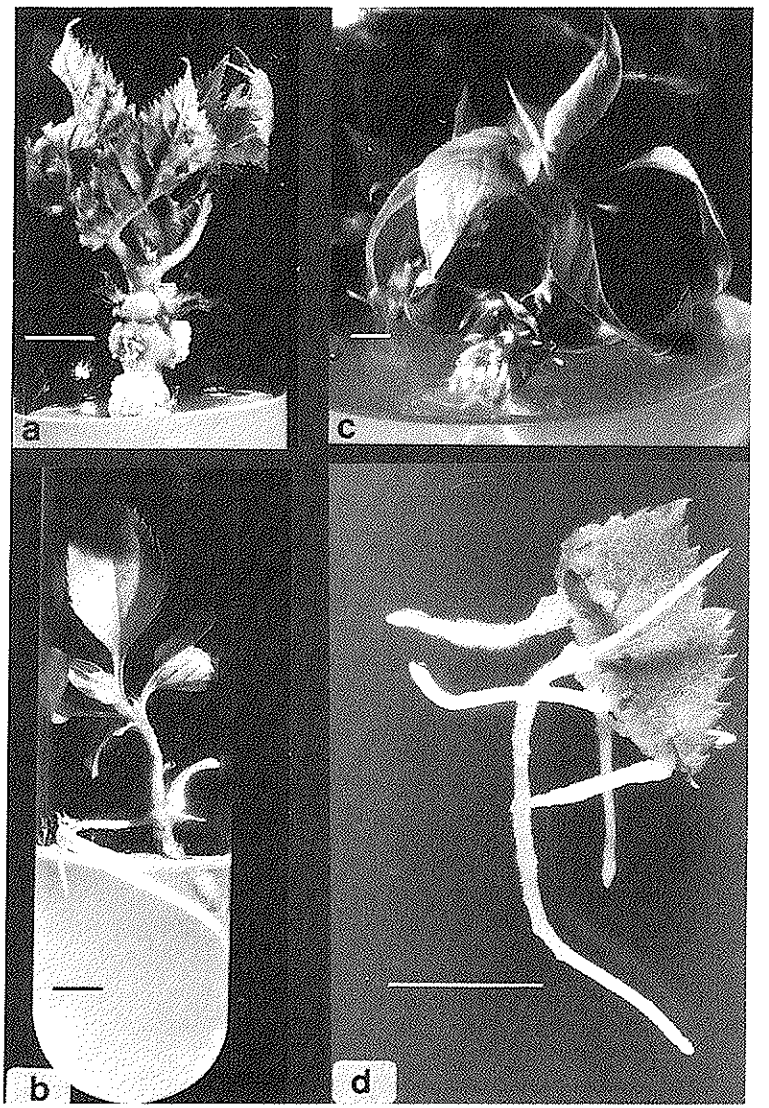

Figure 5. Genetic chimaeras of fruit trees produced after infection with Agrobacteria. (a) Gall formation on stems of cherry rootstock Colt, after inoculation with *A. tumefaciens*, T37. (b) Transformed roots growing from infection site after inoculation with *A. rhizogenes* LBA 9402 in apple cultivar Greensleeves. (c) As (b) using pear cultivar 'Conference'. (d) Transformed roots emerging from cut apple leaf (cv. Greensleeves) after 20 min co-cultivation with same strain of *A. rhizogenes*. Bar = 0·5 cm.

a positive result. Plants transferred to pots grew normally and had no obvious phenotypic changes. The fact that they could be induced to form sufficient adventitious roots *in vitro* for soil transfer suggests that the plants produced in this way are untransformed regenerates. The galls themselves usually consist of both transformed and untransformed cells. Cross-feeding of

Table 6. Responses* of various fruit tree subjects to infection with *Agrobacterium tumefaciens* and *A. rhizogenes* carrying wild-type plasmids

Genus and cultivar	*A. tumefaciens*	*A. rhizogenes*	
	Shoots forming galls (%) (T37)	Shoots forming roots (%)	
		pRi1855	pRi8196
Malus			
Cox	+	0	—
Golden Delicious	0	—	—
Greensleeves	+	+	—
M.9	+	++++	++
M.25	+	+++	—
Pyrus†			
Conference	++	+++	—
P657	—	+++	—
P661	++	+++	—
Cydonia†			
QC	0	0	—
Prunus			
Early Rivers	++++	+‡	—
Colt	++++	++	—
Pixy	+++	+	—

Key: ++++>75%; +++ 50–74%; ++ 25–49%; + <25%; 0, no response
* Based on data of James, Murphy and Passey (1985).
† Data of Browning *et al.* (1985)
‡ Gall formation also

untransformed cells with endogenously synthesized hormones of the transformed cells can lead to the regeneration of untransformed shoots.

At the recent Moet:Hennessy meeting in Paris, several other workers presented data on transformation in fruit trees. Viseur (1986), using the pTi T37 plasmid, inoculated pear plants of the cultivars Comice and Conference and obtained a positive response for nopaline in some of the plants that subsequently regenerated from the galls. Although the initial plants displayed aberrant phenotypes, axillary shoots that developed from them were normal-looking. Dandekar and Martin (1986) infected cherry, peach and apricot seedlings with *A. tumefaciens* carrying the broad-host-range plasmid pTiA6. There was a variation in response, in that only 1–20% of cherry seedlings were infectable and formed tumours, whereas 20–40% of peach and 40–80% of apricot seedlings did so. Octopine was found in the transformed tissues.

Similar problems were encountered with Ri-transformed root systems, except that the frequency of regeneration from such roots was extremely low. Attempts to regenerate shoots from transformed root systems of apple and cherry have not met with any great success. In experiments over a 2-year period in which more than 20 media combinations were used, the transformed roots induced on apple rootstock M.9 failed to regenerate more

than two shoots (James, Passey and Marks, 1986). These shoots could not be sustained on the usual micropropagation media. Similar problems were encountered with several pear genotypes (Browning *et al.*, 1985). The cherry rootstock Colt that regenerates readily from normal roots (*see* pp. 56–58) failed to produce any shoots from Ri-transformed roots, suggesting that there may be changes in endogenous hormone physiology after transformation (James, Murphy and Passey, 1985). This contrasts with the ease with which shoots may be regenerated from transformed roots of herbaceous species such as carrot, tobacco and convolvulus (Tepfer, 1984).

Binary vectors—engineered plasmids

Many of the plants regenerated from galls after infection with wild type *A. tumefaciens* plasmids have aberrant phenotypes, presumably because the endogenous growth regulators are being produced in amounts that interfere with the expression of the normal phenotype. For instance, such plants often have thick twisted fasciated stems with very small leaves and have the ability to grow on hormone-free media. Recent molecular techniques have been used to produce 'binary vectors' of *Agrobacterium* for more efficient transformation (e.g. Bevan, 1984). In these, the genes encoding for growth regulator autonomy have been deleted, because they can interfere with the regeneration of normal plants, and are replaced with one or more dominant selectable marker genes. In these so-called chimaeric constructs, a regulatory sequence coding for an opine synthetase gene drives the coding sequence of a bacterial neomycin phosphotransferase (kanamycin kinase, EC 2.7.1.95) gene (NPT II) which confers resistance to the antibiotic kanamycin (Bevan, Flavell and Chilton, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983). Such constructs allow the regeneration of transformed plants that are morphologically normal but which can be grown in the presence of kanamycin. In the long term this means that existing cultivars may have genes added somatically without any other changes to the phenotype.

Already these constructs are being used to transform fruit trees *in vitro*. The binary vector Bin 6 (Bevan, 1984) has been used by James and Passey (1986) to transform leaf discs of the apple cultivar Greensleeves. About 40% of leaf discs produced nopaline-positive kanamycin-resistant callus (*Figure 6*) whereas control 'disarmed' plasmids lacking the resistance gene failed to produce any callus. Subsequent regeneration of shoots on kanamycin-containing medium occurred at a frequency of 1/50 calluses but these plants need to be screened by Southern blot analyses for unequivocal evidence of transformation.

The Ri phenotype

Tepfer (1983) has comprehensively reviewed the phenotype of Ri-transformed plants in herbaceous species and the sort of changes in rooting behaviour that may be observed. In the aerial parts of Ri-transformed plants the leaves

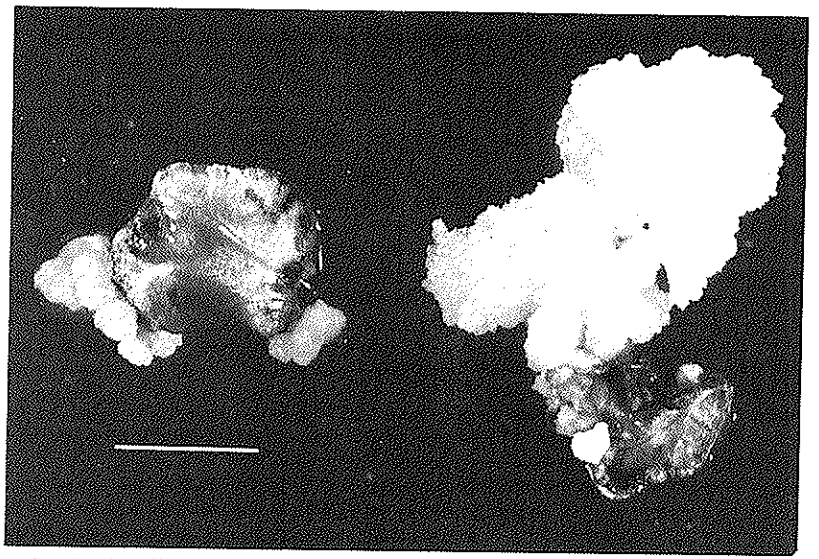

Figure 6. Apple callus on leaf disc, transformed by co-culturing with disarmed binary vector, Bin 6 and growing on 100 mg/l kanamycin. Callus gave a positive response for nopaline as detected by paper electrophoresis. Bar = 0·5 cm.

are wrinkled and shoot apical dominance is greatly reduced, causing much branching; in biennial species (e.g. carrot, endive) there is a change in the annual flowering habit. The root system is changed fundamentally in its morphology, biochemistry and physiology. The transformed roots have faster growth rates, are highly branched and tend to adopt a plagiotropic (oblique) growth habit. The greater root biomass may therefore alter the physical characteristics of the root system by giving greater anchorage and conferring greater drought resistance compared with normal untransformed root systems (Moore, Warren and Strobel, 1979). In most cases the roots produce opines, the type being determined by the original bacterial strain used.

Although transformed plants may have reduced male gamete formation leading to reduced seed set, this is of little concern in predominantly asexually propagated, outbreeding species. Where a breeding component is relevant it should be added that the Ri phenotype is inherited as a single dominant gene (Constantino *et al.*, 1984; Tepfer, 1984) and the character can be selected for at the *in vitro* stage (Tepfer, 1983). For fruit tree rootstocks the potential advantages of increased anchorage and drought tolerance should outweigh any breeding disadvantage. Increased anchorage due to a more extensive root system is an extremely important attribute that it would be desirable to introduce into apple rootstocks such as M.9, which otherwise have excellent rootstock qualities. The growth of scion varieties on Ri-transformed roots could also be attempted in order to assess effects on vigour.

By injecting untransformed shoots with *A. rhizogenes* strains and permitting transformed roots to grow from the infection site, genetic chimeras may

be produced (*Figure 5*) while regeneration from transformed roots would yield wholly transformed plants. Strains of *A. rhizogenes* with the kanamycin resistance gene are now available and enable the selection of Ri-transformants at an early stage. The marker should also permit an assessment of the proportion of the total root system that is transformed once plants have been transferred to the field.

Other strains of *A. rhizogenes* harbour plasmids that have had their agropine-coding genes deleted (F. Casse-Delbard, personal communication). Transformed roots produced by this plasmid should not exude opines into the soil. It remains to be established whether such exudation is important in changing the bacterial ecology of the rhizosphere.

Potential applications and advantages of the Ri phenotype

The changes that may be expected in tree species with Ri phenotype could have considerable benefits to the temperate fruit tree industry:

1. Difficult-to-root tree subjects may be made to root *in vitro* or even directly *in vivo* after producing genetic chimaeras by inoculation with *A. rhizogenes* (Lambert and Tepfer, 1986);
2. The production of totally Ri-transformed plants could mean the introduction of new variants of important commercial fruit tree rootstocks in a very short time;
3. Faster root growth rates may mean faster shoot growth rates and increased survival of young transformed plants due to better anchorage and water stress tolerance.

At the same time there are a number of unknown factors relevant to eventual performance, e.g.:

1. As there are effects on the aerial parts of the plant, will there be changes in branching pattern and how might this affect canopy form and photosynthetic efficiency?
2. How will vegetative and reproductive vigour be affected and will any changes be advantageous?

Such questions can be answered only after conducting the necessary field trials to assess performance.

Which genes?

Having shown that it is possible to put 'foreign' genes into temperate fruit trees we now have to ask which genes we would want to introduce. The conventional fruit breeder will argue, and quite rightly, that many of the most important tree fruit characters, e.g. vigour, yield, storage qualities, fruit flavour and texture, are polygenically controlled. At the moment, and with the notable exception of the 'Ri genes', there are very few genes

available that are likely to make a significant contribution to fruit tree biotechnology. However, progress in DNA recombinant technology is rapid and perhaps one of the most powerful aspects of genetic transformation involving *Agrobacterium* is the ability to transfer genes to all genera of the Dicotyledonae: a given gene transferred and expressed in tobacco one week could, in theory, be transferred and expressed in an apple shoot the next. For example Comai *et al.* (1985) have been able to transform tobacco plants with the gene encoding for an altered and resistant form of the enzyme 5-enol-pyruvylshikimate-3-phosphate synthase (EPSP synthase or 3-phosphoshikimate 1-carboxyvinyl transferase, EC 2.5.1.19), that is the major target for the herbicide glyphosate. This '*aroA*' gene was originally isolated from glyphosate-resistant *Salmonella typhimurium* and recombined into *A. rhizogenes* plasmids where it was used to transform leaf discs. Transformed plants showed only partial tolerance to applications of the herbicide, leading Comai and colleagues (Comai *et al.*, 1985) to suggest that there is more than one site of action of the herbicide. With the development of suitable regeneration techniques for fruit trees, this gene, or tailored versions of it (Shields, 1985), could be transferred into the desired species. In fact the gene has already been transferred into the tree species *Populus* and its expression has been confirmed (Fillatti, Sellmer and McCown, 1986).

Another currently available gene is that cloned from *Bacillus thuringiensis* which codes for a crystalline protein that is toxic to a wide range of lepidopteran species (Dean, 1984; Adang *et al.*, 1986). Expression of the gene in host leaf tissues is fatal for caterpillar larvae feeding on such leaves. So far this gene has been expressed in tobacco but this could be extended to fruit trees for protection against a wide range of economically important insect pests. The lack of toxicity to mammals and birds that ingest the protein is an added attraction.

The ability to clone the gene for the compact habit in apples would have far-reaching applications because it could be transferred to any other fruit tree species for which regeneration protocols have been described. Unfortunately, the mechanism controlling this habit is not understood and the basic physiological and biochemical work is needed to identify and manipulate the gene concerned. The same could be said of genes that control fruit taste and flavour, or of those controlling juvenility or a host of other important horticultural characters.

Conclusions and future prospects

Although temperate fruit trees rank with other crops such as potatoes and pulses in production and world trade values, the amount of published work on their tissue culture and somatic cell genetics is disproportionately small.

A point that requires re-emphasis is the need to control the rejuvenation effect that some fruit tree cultivars experience after micropropagation. Unless this can be done we must expect the effects of *in vitro* genetic manipulation to be superimposed on those of rejuvenation. Induction of rejuvenation could be useful under certain circumstances: for example, some otherwise

valuable rootstocks such as the apple rootstock M.27, may be too dwarfing and might benefit from more vigorous growth.

Haissig (1987) has concluded that somatic embryogenesis may eventually replace micropropagation for the rapid propagation of forest trees. In these species juvenility may be advantageous in that it should serve to promote rapid growth with assimilates being diverted into vegetative rather than reproductive vigour. As the opposite usually applies in fruit trees, the development of somatic embryogenesis as another propagation system may have limited application even if the tissue culture methodology can be defined, developed and shown to be more efficient than micropropagation. Exceptions to this are cases where somatic embryogenesis can meet specific breeding objectives by rapidly multiplying germplasm that is initially present as embryonic material, e.g. maternal embryos, haploid embryos and interspecific hybrid embryos that normally abort through endosperm failure.

In vitro mutagenesis and somaclonal variation may well have similar roles in producing new variants of existing valuable commercial varieties, but because they will be derived from essentially uncontrollable sources of variation they will demand considerable time and effort to screen regenerated plants for the trait(s) of interest. *In vitro* mutation studies are ideal where a simple pattern of inheritance for the trait of interest is known. Somaclonal variation could be valuable to correct for particular character deficiencies but usually those of greatest interest (e.g. fruit characters of scions, burrknotting, suckering of rootstocks) are manifest long after the tissue culture process. Disease resistance could be an exception, where suitable screens can be imposed that are based on sound biological knowledge of the host–pathogen relationship. The production of disease-resistant somaclones of fruit trees will probably be one of the first claims of the new technology, although the persistence of the character will need testing over many propagation cycles. The need to develop *in vitro* screening procedures is greater in tree species than in any other group of plants because of the space and time needed for the evaluation of new variants.

The development of this methodology requires an understanding of the mechanisms that lead to the deficiency in the character to be improved. Usually, this basic information is not available.

The capacity of tissue culture technology to produce non-cyto-chimaeral polypoloids may, in the short term, be one of its most suitable and valuable applications. Such polyploids should be used as 'ploidy bridges' in conventional breeding programmes to overcome problems of sterility and self-incompatibility.

The first report of success in regeneration of a temperate fruit tree species from protoplasts should prompt the extension of this to many other species over the next 5–10 years. It can therefore be expected that somatic hybrids of both fundamental and applied interest should be available for field trialling by the turn of the century or perhaps even earlier. Concurrent developments in molecular genetics should see a range of dominant biochemical and physiological marker genes available for early selection of heterokaryons. These will parallel and complement other selection methods based on fluor-

escence-activated cell sorting. At first, intrageneric fusions involving rootstocks and scions should be attempted because these are likely to be more viable and probably of more value to the agronomist and, eventually, to the grower.

The short-term role of genetic transformation in fruit tree improvement is uncertain. As with somaclonal variation, it has arrived in advance of a more complete understanding of the physiological and biochemical mechanisms that control fruit tree growth and development. It would be very difficult to find a more graphic illustration of this than the *Co* gene in apples. This is a wonderful target for genetic engineering but our attempts to transfer the gene to other cultivars and other fruit tree species will be frustrated until we understand the mode of action of the gene and how it is expressed in the phenotype. These deficiencies in our understanding of basic mechanisms that control plant growth and development will prevent us from taking advantage of the opportunities offered by molecular biology.

It is probable that Ti- and Ri-transformed fruit trees will become available in the next few years. Trees transformed with wild-type plasmids may or may not have direct field use but they should certainly add valuable clues to the ways in which shoot and root growth are controlled. *Agrobacterium*-mediated transfer of other horticulturally important genes can be expected to follow, as and when these become identified and available.

The term 'genetic manipulation' should be taken in its widest context; simple techniques such as the induction of ploidy changes may be equal in importance to the transfer of genes from one crop to another. For instance, an apple variety with the Wijcik gene in the homozygous dominant condition would probably be of greater importance to the breeder (and to the industry) than a herbicide-resistant tree.

This review may more accurately be described as a preview, as the use of tissue culture technology on fruit trees is still in its infancy. However, the Moet:Hennessy meeting on Fruit Tree Biotechnology in 1986 has revealed the enormous potential available for fruit tree improvement. Nevertheless, potential is distinct from practice and many of the future applications of the new technology are still uncertain. What is certain is that there must be a continuing dialogue between molecular biologist, cell biologist, physiologist, pathologist, breeder and agronomist to identify suitable and sensible targets for genetic manipulation. Without this dialogue there will be a great deal of misdirected and expensive research.

Postscript

Since this review was completed, Ochatt, Cocking and Power (1987) have successfully regenerated whole plants from mesophyll protoplasts of the cherry rootstock Colt. This marks the first report of plant regeneration from protoplasts of a temperate fruit tree species of significant commercial value.

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