MICROPROPAGATION AND *IN VITRO* MUTAGENESIS FOR CROP IMPROVEMENT

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INTRODUCTION

Plant tissue culture has application in the production and cloning (micropropagation) of elite disease-free material and in the provision of efficient experimental systems for plant genetic manipulation. A pre-requisite for the use of tissue cultures is the establishment of aseptic (contaminant-free) cultures. Micropropagation requires not only the maintenance of clean cultures but also the maintenance of genome stability and avoidance of epigenetic variation. Plant genetic manipulation, which is a blanket term for non-conventional plant breeding methods, seeks to generate variability. This variability that shows genotype-dependence, can arise spontaneously in shoots arising adventitiously from explants and callus, particularly when caulogenesis occurs in callus derived from polysomatic tissues. Spontaneous variation in tissue culture derived plants is termed 'somaclonal variation' (for a recent review on this topic see Karp, 1991). Where it occurs, somaclonal variation may be amplified by mutagenesis *in vitro*. Genetic transformation whether *Agrobacterium*-mediated, or following electroporation or particle bombardment, is conducted at the cell level and unwanted background somaclonal variation may also result. Similarly, somaclonal variation may arise in protoplast culture. The problems of contamination control and unwanted genetic variation are thus common to micropropagators and to those using genetic manipulation. Tissue cultures may also be used in attempts to increase the efficiency of genetic manipulation. Markers incorporated into constructs may be used to locate or select transformed cells, or biotic stress applied *in vitro* may increase the efficiency of selection of potentially useful mutants/somaclones.

Here, some selected developments in plant tissue culture and in *in vitro* mutagenesis are discussed in relation to improving the efficiencies of these technologies. More comprehensive reviews of micropropagation and plant genetic manipulation are to be found in Debergh and Zimmerman (1991), George (1993); and in Cassells and Jones (1995), respectively.

PRODUCTION OF ASEPTIC CULTURE

It is difficult to prove that cultures are axenic, that is, free of all microbial contamination. Here the term aseptic is preferred to denote that cultures are free of the specific diseases for which they have been indexed (Cassells, 1991).

It is now generally accepted that contamination of plant tissue cultures can be ascribed to two sources, namely, contamination carried into culture on or in the explant; and secondly, contamination arising in the laboratory from environmental or human-associated bacteria due to faulty working practices (Cassells, 1992).

(a) Establishment of aseptic cultures

Meristem-tip culture is recognized as an efficient way of elimination bacterial and bacteria-like agents from plants (Walkey, 1991). The principle is that the meristem-tip escapes contamination affecting the soma of the plant. In the case of phloem-restricted organisms, the explant taken should be excised beyond the region of vascular development. As a generalization, the smaller the explant the more stringent are the nutrient requirements. Bacterial nutrients may be incorporated into the meristem culture medium to encourage the expression of bacterial contaminants (Menard *et al*, 1985). Where meristem-tip culture is not effective in eliminating bacterial contaminants, specific measure may be taken. Donor plants may be treated with antibiotics (Cassells *et al*, 1988) or contaminants expressed in culture may be isolated in pure culture, their antibiotic sensitivity determined and the antibiotic incorporated into the medium in further rounds of meristem culture (Barrett and Cassells, 1994).

Excision of the meristem-tip may also eliminate viruses and viroids (Walkey, 1991). Traditionally heat-therapy has been used to eliminate viruses (Walkey, *loc. cit.*), however, the antiviral (virist-atic) compound Ribavirin (syn. Virazole) has broad spectrum activity, including reported activity against viroids, and is effective in plant tissue cultures provided that the explants are cultured in the continuous presence of Ribavirin and that only the new growth is excised (Cassells and Long, 1982).

These strategies are summarized in Figure 1.

(b) Monitoring for laboratory contamination

Provided that rigorous screening for contaminants is carried out on the initial cultures and that aseptic cultures have been established, then the multiplication of cultures for '*in vitro* biomass' for genetic manipulation or for micropropagation can proceed. The risk is from

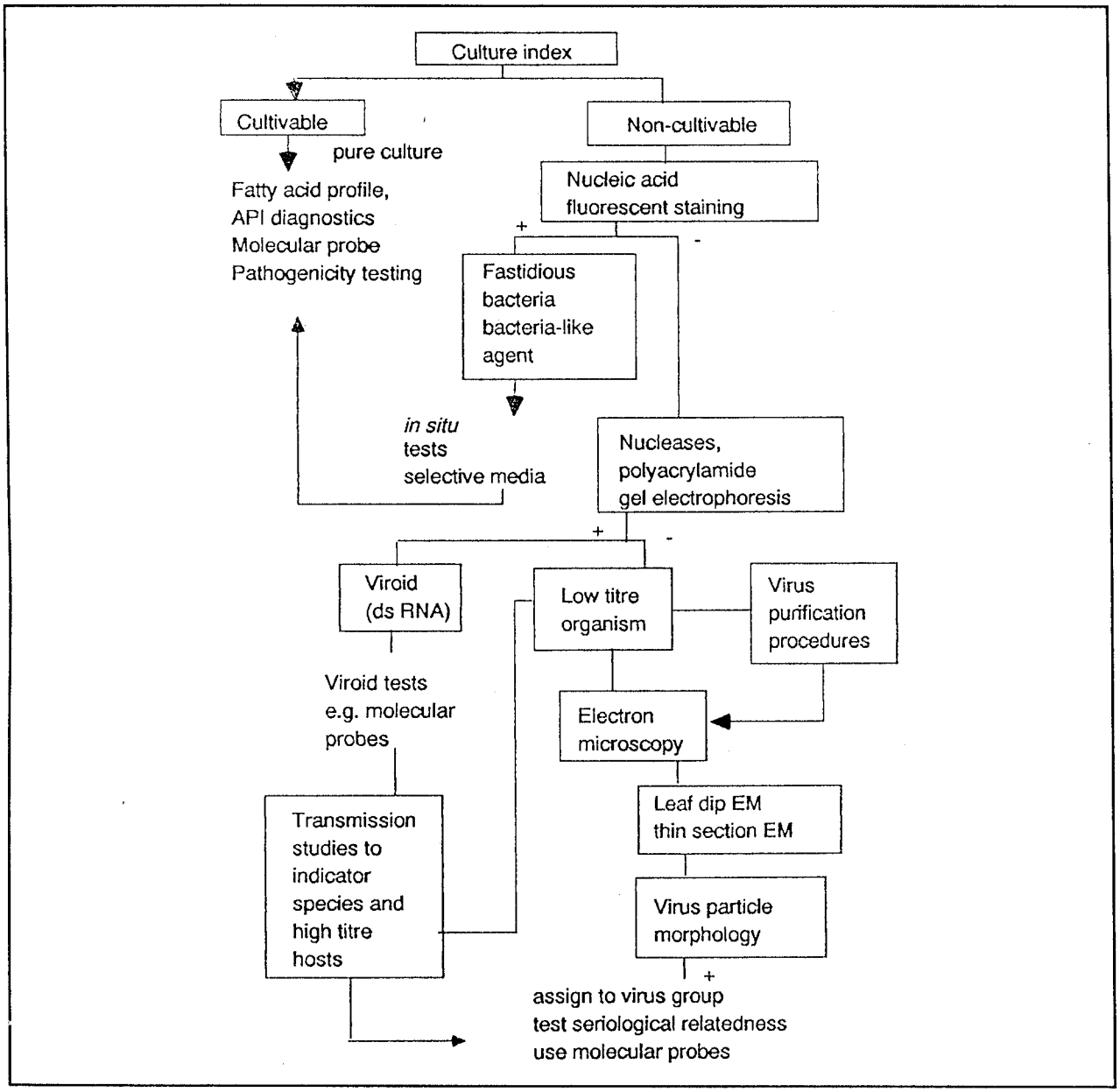

Figure 1. A scheme summarising strategies for the production of elite disease-free plants. Following the establishment of aseptic disease-free cultures, these can be multiplied *in vitro* or microplants can be established and multiplied *in vivo*. During *in vitro* multiplication there is the risk of contamination by environmental or human-associated cultivable bacteria (see text); multiplication in the environment involves the risk of exposure to pathogen inoculum (from Cassells, 1995).

laboratory contaminants entering as a consequent of system failure e.g. faulty sterilization or operative error (Leifert and Waites, 1994). While visual examination of cultures may detect contamination, it is essential to back this up with culture indexing for cultivable bacteria. The cultures may be sampled assuming a Poisson distribution for the contamination but, once foci of infection have been detected, its advisable to screen up- and down-stream of the contaminated culture to confirm the limits of the infection focus (Cassells 1991).

(c) Clean stock production

Aseptic culture may be used to produce elite disease-free nuclear stock for multiplication in the environment or aseptic cultures may be used directly to produce disease-free planting material. The difference between the two strategies is illustrated in Figure 1. Multiplication *in vitro*, it is argued, is less likely to result in contamination by pathogens, due to the absence of pathogen inoculum in the laboratory; whereas this risk is potentially high when multiplication is in the environment. There is a risk of laboratory contamination during *in vitro* multiplication that may result in serious losses if good working practice is not observed (see: review of laboratory good working practice by Leifert and Waites, 1994).

DEVELOPMENTS IN MICROPROPAGATION SYSTEMS

In addition to the problems presented by contamination, the micropropagator may have problems also with the quality of biomass *in vitro* and with the poor performance *ex vitrum* of such material. Abnormal *in vitro* morphology, termed 'vitrification' or 'hyperhydricity' (Ziv and Ariel, 1994), can result in low multi-plication rates; microplants may require costly weaning facilities e.g. fogging units, and subsequent habit abnormalities, such as loss of apical dominance, may reduce the crop value. Vitrification has been extensively reviewed and the cause ascribed to media factors and the container design (see e.g. Ziv and Ariel, *loc. cit.*, and references therein). Vitrification has been ameliorated or eliminated by using bottom cooling (Maene, 1989) in sealed containers or by using permeable membranes as culture vessel lids (Cassells and Walsh, 1994; Cassells and Roche, 1994). Investigation suggests that the vitrification syndrome is associated with low calcium accumulation in the tissues of microplants grown in high humidity environments *in vitro*. Bottom cooling, or the use of lids which allow controlled water loss, and consequently reduce the vessel humidity, increases the uptake of calcium in the microplants, by transpiration, and results in the development of functional stomata which resist desiccation of the microplants at establishment (Cassells and Roche, 1994).

Induction of a transpiration stream in the shoots of microplants has been shown to result in the suppression of vitrification and improved *post vitrum* performance. Roots produced in solidified media, however, are also abnormal. Agar has several negative characteristics (e.g. Kohlenbach and Wernicke, 1978). Firstly, it may vary in composition and purity from batch to batch; secondly, agar may bind minerals and so the available ion concentration is unknown, and thirdly, oxygen supply to the roots is restricted. Recently, membrane rafts have been introduced to overcome these problems. In this laboratory we have adopted the use of plastic foams are alternatives to agar, with significant improvements in biomass quality and *post vitrum* performance (Cassells and Walsh, in press).

SCALING UP IN MICROPROPAGATION

It remains to be demonstrated unequivocally that genome stability can be maintained in the multiplication stages in large scale somatic embryogenesis on which synthetic seed production is based (Durzan and Durzan, 1991). For many vegetatively-propagated crops e.g. potato, pelargoniums etc. There are contra indications. In the interim, and for unstable genomes, alternative scale-up strategies, that is cost reduction strategies, are required for micropropagation to remain competitive in the 'high-cost' economies. Photoauto-trophic micropropagation offers one option (Aitken-Christie, 1991). In the latter, production of aseptic cultures and production of 'seed cultures' follows the stages of conventional heterotrophic culture. The 'seed' cultures, however, rather than being continuously subcultures in conventional vessels, are used to seed large vessels which are provided with simple mineral solutions lacking organics and sucrose. These are incubated in CO^2-enriched growthrooms with elevated light

levels, following the principles developed by Kozai (1991). Such systems have been in production in Ireland for potato since 1990 and, more recently, have been set up in South Africa.

MONITORING GENETIC FIDELITY IN MICROPROPAGATION

It is recognized that the risk of genetic drift in micropropagation is associated with the cloning strategy employed and is genotype dependent (George, 1993). Nodal culture is low risk, meristem cultures varies from low to high risk depending on whether proliferation is *via* precocious axillary bud proliferation of *via* a mix of axillary and adventitious bud proliferation; whereas adventitious regeneration, especially *via* a callus phase, is high risk (George, *loc. cit.*). The problem for the micropropagator is an economic one, namely, the time/cost required to confirm varietal identification and trueness-to-type. This depends on sampling the population and, in the case of flowering plants, growing on to flowering. In practice, this criterion was rarely, if ever, met.

Molecular techniques, in theory offer solutions to the problem of confirming varietal fidelity but are expensive, require skilled labor and may be difficult to set-up. Measurement of the standard deviation of a quantitative trait has been advocated as a way of detecting increased variability in a population e.g. somaclonal variation (De Klerk, 1990). Numerical taxonomic methods, based on data acquisition by image analysis, interfaced with statistical analysis, have proved to be a powerful cost effective method both for the determination of varietal identify and genome variability (Cassells *et al*, 1993).

IN VITRO MUTAGENESIS

The theory and practice of mutagenesis has been discussed in detail elsewhere (Anon. 1977; Broertjes and van Harten, 1988). Here, consideration will be given to a comparison of the relative merits of intact buds (apices) versus cells (in complex explants) as targets for mutagenesis, in the context of developing costeffective strategies for the application of this technique, with emphasis on the improvement of vegetatively-propagated crops.

(a) A comparison of buds versus complex explants as targets for mutagenesis

In the early years of mutation breeding, mutagenic treatments were applied to seed or, in the case of vegetatively-propagated plants, to whole plants or cuttings (Broertjes and van Harten, 1988). Chimeras resulted that were difficult to detect in many cases i.e. non-phenotypic chimeras, and that were unstable. In the case of fertile species, chimeras could be eliminated, as other defects by back crossing to the parent; in the case of outbreeding, polyploid, vegetatively-propagated crops this was not possible without massive segregation and consequently, selection was slow and expensive. Furthermore, whole plants or cuttings, as opposed to seed, required large facilities for treatment etc. Tissue culture has revolutionize mutagenesis in providing miniaturized propagules i.e. a high density of target cells/apices can be provided for experimentation. Broertjes (1982), advocated the use of complex explants as the targets for mutagenic treatments, on the basis that mutations are cellular events and that adventitious shoots are normally regenerated from single cells. Thus mutations can be expressed directly as homohistonts. Irradiation of buds *in vitro*, as *in vivo*, results in the problem of chimerism. The Broertjes (1982) model has gained wide acceptance. In opposition to this model, Sonnino *et al.* (1986) treated apices *in vitro* with physical mutagens, passaged the treated buds through subculture cycles to breakdown the putative chimeras and selected for variation in the progeny. Their experiments were not completely successful in that some chimeric progeny were detected, possibly because too few subcultures were used. Cassells *et al.* (1993) further evaluated this strategy in attempts to produce a flower color family in a sterile *Dianthis barbatus* x *D. caryphyllus* hybrid (see also Cassells and Periappuram, 1993), using irradiated fragmented apices as controls.

In the case of the irradiated complex explants, the population of adventitious regenerants was very variable in morphology and vigor, with a number of mutants showing darker or paler flower colors than the parent. In the case of the irradiated buds, after three cycles of subculture *in vitro*, the population showed no phenotypic variability in the field, although on flowering, mutants with lighter and dark flower color than the control were detected. Lines differing in susceptibility to *Alternaria* Blight were also detected (Cassells *et al*, 1993).

In the case of *Dianthus*, irradiation of complex explants results in a highly variable population, whereas in the population derived from irradiation of apices, which had undergone diplontic selection, only 'fit' mutant cells survived to give rise to homohistonts. In the latter, it is hypothesised, competition between mutated and unmutated cells in the histogenic layers of the apical meristem (Cassells and Periappuram, 1993) resulted in the elimination of unfit mutant cells. Thus if high variability is sought, irradiation of complex explants is advised; if correction of a character defect is the objective, to be combined with retention of agronomic performance, then irradiation of apices, with diplontic selection may be more efficient but it carries the risk of 'over-selection' for 'fitness'.

(b) *In vitro* selection for disease resistance

In vitro selection for disease resistance has been reviewed by Jones (1990). Here, three model studies will be discussed to illustrate some of the problems associated with selection for resistance and some possible solutions. While specific pathogen/models differ in their details, some basic points can be made. Firstly, many of the factors which contribute to host resistance, e.g. cuticular waxes, cell wall lignification etc. may not be expressed in the *in vitro* cells or tissues used for selection. Secondly, pathogen metabolites that may accumulate in *in vitro* cultures may be toxic to plant cells and tissue *in vitro* but may not be determinants of pathogenesis *in vivo* (Jones, 1990).

(i) Selection on toxin-specific media

In the work on *Dianthus* flower-colour family referred to above, mutants with altered resistance to *Alternaria* blight were isolated in field trials (Cassells *et al*, 1993). *Alternaria* produces a host specific toxins (Frisvad and Thrane, 1993) and so, to validate an *in vitro* selection scheme for resistance to *Alternaria*, selected mutants, varying in disease resistant, were re-introduced into culture and subcultured on media on which *Alternaria* had been grown then removed prior to inoculation with the plant tissue. The results showed a significant correlation (r = -0.98) between field resistance to the pathogen and growth rate on pathogenmetabolite containing medium.

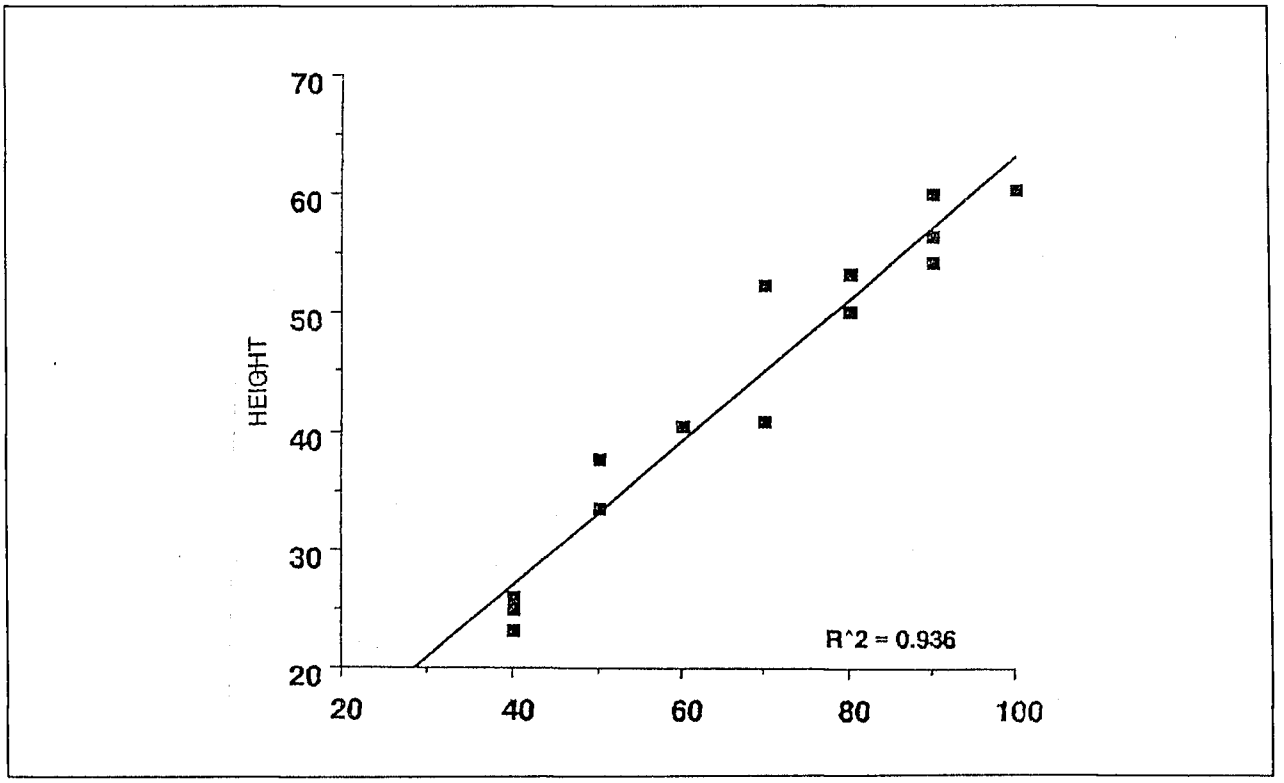

Figure 2. The correlation between inhibition of growth of nodes of *Helianthus tuberosus* cultivars/lines *in vitro* on low calcium medium and disease severity in the field (from Cassells and Walsh, 1995).

(ii) selection for ability to grow on low calcium medium

Sclerotinia sclerotiorum causes a serious basal stem rot of *Hellanthus tuberosus*, unlike *Alternaria, Sclerotinia* does not produce a pathogen-specific toxin (Cassells and Walsh, 1995). Like many fungi and bacteria, *Sclerotinia* produces chelating agents, in this case oxalic acid, which, with cell-wall degrading enzymes, are among the pathogenicity factors. Attempts, as above, to demonstrate the different levels of resistance expressed by *H. tuberosus* varieties and mutants in the field, by culturing tissue in medium pre-colonized with *Sclerotinia* failed to show any correlation. Oxalic acid interacts with host cell and membrane calcium in the infection process (Cassells and Walsh, 1995), so it was decided to reverse the selection and select for the ability of host varieties and mutants to grow on low calcium. The rationale was that varieties/lines that could grow on low calcium, might accumulate higher levels of calcium *in vivo* and be resistant to the pathogen. A correlation was found between ability to grow on low calcium *in vitro* and resistance to *Sclerotinia* in the field (Figure 2; Cassells and Walsh, *loc. cit.*).

(iii) resistance to late potato blight

Late potato blight has re-emerged as the main disease of potato world-wide (van der Zaag, 1995). This is a consequence of the spread of the A2 mating strain resulting in more variability in the pathogen, including more fungicide resistance; and due to the imminent withdrawal of important fungicides on environmental grounds. Toxic fungal metabolites have been used as an *in vitro* selection agent for blight resistance (Behnke, 1980) but the progeny plants did not survive a field trial. Attempts at pathogen/host co-cultivation *in vitro* have also been unsuccessful (Meulemans *et al*,1986). In a long-term programme in this laboratory, on the evaluation of somaclonal variants and of the progeny of irradiated nodes and explants, attempts to isolate improved blight resistance lines have focused on a strategies aimed at the early detection and elimination of useless variants e.g. polyploids, aneuploids and maturation mutants that characterize adventitious regenerants of potato (Cassells *et al*, 1991; Cassells and Shen, 1995). While phenotypic aberrants e.g. polyploids and dwarfs etc. can be selected by eye, image analysis has the potential to detect maturation mutants also (Cassells and Sen, *loc. cit.*). This category of useless variants is important, as late blight resistance is associated with late maturation. These mutants express improved resistance in early field trail but revert (Cassells *et al*, 1991). If they are allowed to progress in the trials they can be a cause of considerable waste expenditure.

CONCLUSIONS

Here, some of the factors which have limited the application of plant tissue culture in micropropagation and genetic manipulation have been discussed, for more comprehensive reviews see Debergh and Zimmerman, (1991) and George (1993). Generally applicable methods now exist for pathogen elimination, aseptic multiplication and the production of true-to-type progeny but caution must be exercised and good working practice followed (Cassells, 1995). Two areas of continuing concern are, uncertainty in many cases as to the pathway, or pathways, of regeneration in relation to the maintenance of genome fidelity; and secondly, epigenetic effects resulting in altered plant habit e.g. hyperjuvenility, altered branching pattern etc.; both types of variation may affect the price of tissue-cultured plants and cause unwanted background variation in plant genetic manipulation experiments. Image analysis appears to have a role to play in monitoring genome fidelity. Improvements in container design and in the use of inert supports e.g. plastic foams, and the use of photoautotrophic systems, may encourage normal habit development *ex vitrum*.

The application of mutagenesis (see review by Maluzynski *et al*, 1995) including the exploitation of somaclonal variation, to plant improvement has been the subject of much research. Tissue culture systems have improved the efficiency with which mutagenesis can be carried out (Figure 3). Nevertheless, there remains the problems of detection and rejection of useless variants which otherwise clutter-up trials programmes; and the early detection of potentially useful variants which can then be multiplied for yield and character trials. Image analysis has been shown to have application in the detection of some useless variants and can be used to quantify population variability (De Klerk, 1990, Cassells and Periappuram, 1993). *In vitro* screening may also have a role in selection for biotic and abiotic stress resistance but with the caveat that ma-

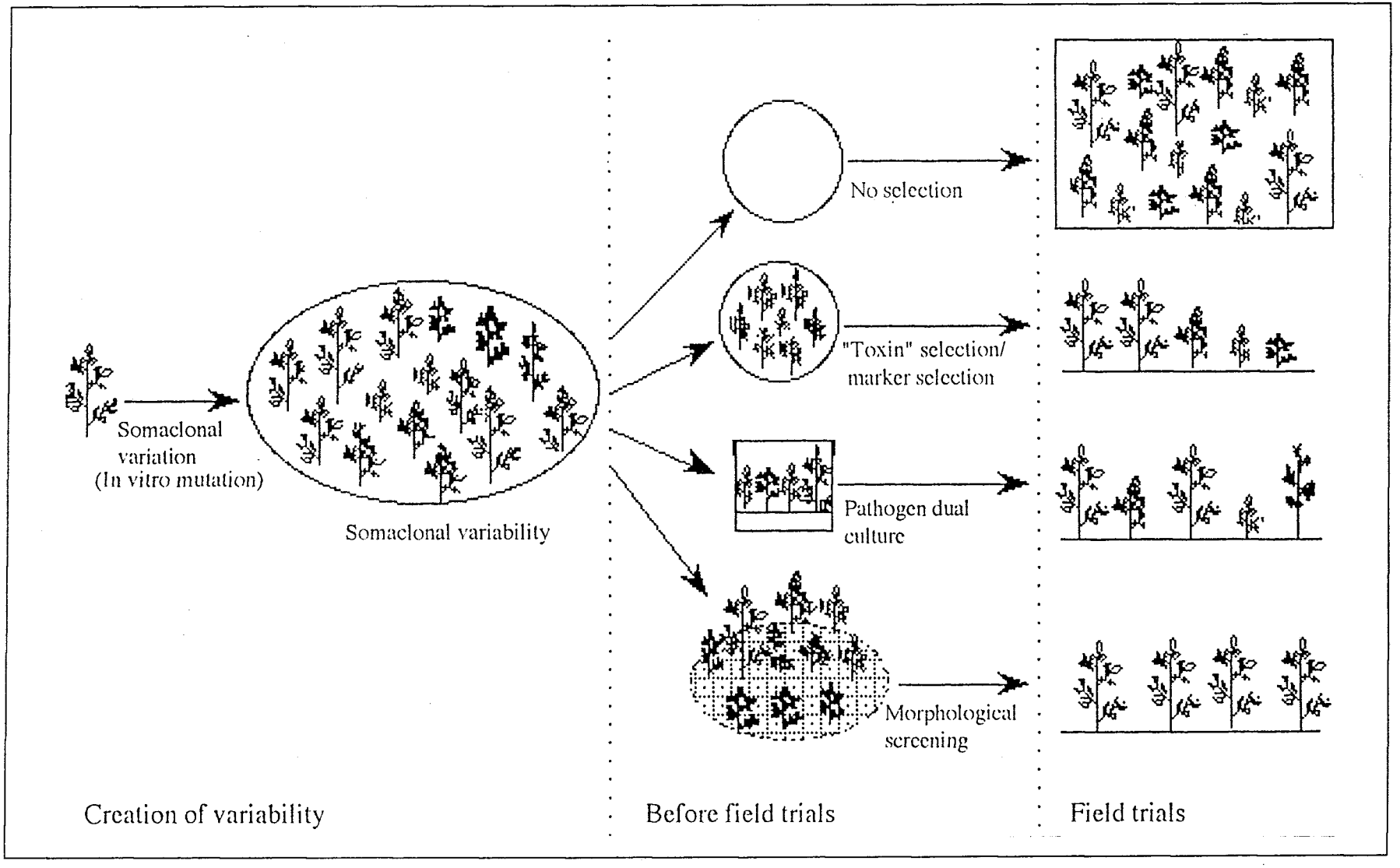

Figure 3. A representation of the use of tissue culture in mutation breeding for disease resistance. Creation of variability may be through spontaneous mutation (somaclonal variation) or induced mutagenesis by e.g. x-irradiation. If adventitious regenerants are transferred directly to the field without selection, the trial will contain a high proportion of uselesss variants. If 'toxin' selection is used, then degenerate lines may pass through to the field (see Behnke, 1980); similarly, if pathogen dual culture is used (Meulemans *et al.* 1986). Where no specific *in vitro* selection is possible, off-types can be selected against at microplant establishment and only plants with normal phenotypes planted in field trials (Cassells and Sen, 1995; see text also).

ny characters are not expressed at the cell, callus or microplant level (Jones, 1990). Disease resistance is a major target in plant breeding programmes and due to the vulnerability of major genes for resistance, new strategies for creating durable resistance are being sought (Johnson and Jellis, 1992). While resistance based on gene-stacking by transformation may have an important future role, in the short term, mutagenesis has an application (Cassells and Jones, 1995). Physical mutagenesis can affect more than one change in a target cell and frequently mutations are expressed as gene dose effects e.g. the flower-color series in *Dianthus* described above. The hypothesis on which calcium selection was carried out was that alteration in constitutive expression of a gene(s) of calcium metabolism might have associated pleiotropic consequences with regard to pathogen resistance. Equally, multiple mutation could result in the over- or enhanced-expression of other constitutive determinants of host resistance to disease in mutated cell progeny e.g. over-producers of phytoalexins, cuticular waxes etc. Such mutants could be selected and might, it is hypothesized, show polygenic disease resistance. This remains to be confirmed.

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