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GENETIC VARIABILITY IN TISSUE CULTURE: IMPACT ON GERMPLASM CONSERVATION AND UTILIZATION

by

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Cover photo: Carrot embryo in culture (L.A. Withers)

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1. Introduction

The ever increasing pressure of an expanding world population on limited food, fibre and industrial crop productivity requires more efficient plant breeding. The expected but often unpredictable perturbations in climatic and edaphic factors, disease, consumer demands and political will means that plant breeding must also be a continuing enterprise.

The fundamentals of plant improvement are genetic variability, recombination, genotype selection and evaluation. Without genetic variability there is no transgressive segregation resulting from recombination and genetic assortment, and therefore no opportunity for genotype improvement by differential selection. Thus future plant breeding options will depend heavily on adequately conserved, broad spectrum germplasm resources.

During the past decade the IBPGR has had a dramatic impact by organizing and coordinating global efforts on conservation of crop genetic resources. The most widely used method for conservation is by seed storage. However for a number of important crop species seed storage is difficult or impossible. There are several classes of species which fall into this category. First, those species for which domesticated cultivars do not produce seed, e.g. *Musa*. Second, those species which do produce seed but which are highly heterozygous and usually vegetatively propagated. Third, those species classified as having recalcitrant seeds where the seed quickly loses viability under conventional seed storage conditions, e.g. cocoa.

To circumvent these difficulties, *in vitro* methods developed initially for the propagation and genetic manipulation of plant species, have and are currently being adapted for germplasm conservation. An extensive literature has developed on *in vitro* methods for conserving plant germplasm resources (for reviews see Kartha, 1982; Withers and Williams, 1982; Wilkins *et al.*, 1982). An initial, and a revised survey (Withers, 1982), commissioned by IBPGR, provides detailed information on research into methods for *in vitro* germplasm conservation. This has provided a database for information exchange and coordination (Anon. 1983).

The purpose of this report is to comprehensively examine variability arising during *in vitro* culture, particularly that which might affect the genetic integrity of a germplasm resource. The framework of this requires an initial brief discussion of *in vitro* culture in relation to germplasm conservation. This discussion is necessary because the type of explant used and the tissue culture and storage methodologies employed for conservation can influence the extent of tissue culture generated variability.

2. In vitro culture techniques

In vitro culture (IVC) or tissue culture is an all-embracing term which describes the growth and manipulation of plant cells, complete plant organs or parts of them under more or less defined nutrient conditions usually in an artificial environment. For practical purposes all plant explants, be they single cells (protoplasts) or complete organs, derive from *in planta* developmentally regulated processes. *In vitro* systems include the culture of explants which retain developmental integrity, or those for which the culture conditions lead to more or less cell dedifferentiation. The latter process may be reserved by regeneration to produce plants by somatic embryogenesis or organogenesis.

2.1 Organized explants which retain developmental integrity

Zygotic embryos. Either mature or immature embryos excised from the mature or developing grain are cultured to produce one or more plants. It is only where cultured embryos are proliferated that limited dedifferentiation occurs.

Meristems. These can be derived from shoot tips or axillary buds. Proliferation of meristems by appropriate manipulation of growth regulators is usually interpreted as retaining developmental integrity.

2.2 Explants which dedifferentiate

Dedifferentiation is the process where at least some cells of a plant organ "lose" developmental regulation to become more or less rapidly dividing dedifferentiated cells. The consequence of this is an amorphous callus without any apparent morphological discontinuity. Incomplete dedifferentiation leads to a complex callus which is a mixture of both organized and unorganized tissues. Depending on species, callus can be induced from any plant organ including zygotic embryos, seedling structures, stems, leaves, roots, flower parts and fruits. Plant gametophytes, i.e. microspores and ovules, can be cultured *in vitro* to give rise to haploid or, following spontaneous or induced chromosome doubling, dihaploid cultures. Callus colonies with a defined single cell origin can be obtained following the isolation of protoplasts by enzymatic or other means. Suspension cultures, usually derived from callus, are mixed populations of single cells and small cell aggregates.

2.3 Plant regeneration and propagation

Generally speaking, the major advantage of tissue culture is the ability to regenerate plants from callus cultures. In a genetic context, this permits the evaluation in mature plants of genome manipulation under *in vitro* conditions. This is the major driving force responsible for the integration of *in vitro* techniques into plant improvement and the expansion of plant molecular biology research.

The number of plants which can be regenerated or clonally propagated from *in vivo* cultures has risen exponentially during the past decade. Documented lists are provided in reviews by Murashige (1974), Tisserat *et al.* (1980), Evans *et al.* (1981), Conger (1981). The ease and relative efficiency of plant regeneration varies widely. Most often the first report of successful regeneration in a new species has a low level of efficiency. Manipulation of culture conditions and use of different explants can usually enhance regeneration efficiency. With the exception of soyabean and cotton, efficient plant regeneration from tissue cultures of all major crop species has been achieved.

Regeneration from callus cultures and clonal propagation from meristems takes one of two forms. Somatic or asexual embryogenesis is the process of embryo initiation and development from cells that are not products of gametic fusion (Tisserat *et al.*, 1981). The products of somatic embryogenesis are often called embryoids to distinguish them from zygote embryos but this is confounded by using the same term to refer to regenerating structures which resemble a proembryoid, often described as no more than nodule discontinuities in a callus mass. Organogenesis refers to the formation of supernumerary adventitious and axillary buds or the *de novo* formation of meristems. Morphological criteria which can vary between observers are used to distinguish between embryogenesis and organogenesis. Recent morphological and histological studies of

several species indicate a graded pattern of organogenesis. Unipolar meristematic structures with vascular connection to the callus grade through to complete bipolar structures with both a shoot and root meristem (Wernicke et al., 1982; Wicart et al., 1984). It is not uncommon to observe somatic embryos, embryoid like structures and meristems in the same regenerating callus.

3. In vitro germplasm storage

There are two basic approaches to in vitro germplasm storage, namely, minimal growth and cryopreservation. Minimal growth conditions for short to medium term storage can be achieved in several ways. They include maintenance of shoot cultures at a reduced nutritional status, particularly reduced carbon; by induction of osmotic stress with mannitol or sucrose; reduced temperature and/or light; incorporation of sub-lethal levels of growth retardants (see Withers and Williams, 1982; Kartha, 1982). The advantage of this method is that the cultures can be readily returned to normal growth conditions to produce plants on demand. The disadvantage is the need for subculturing every 1-2 years, risk of contamination but most importantly substantial selection pressure is imposed and genetic change can be expected over time.

Cryopreservation at the temperature of liquid nitrogen (-196°C) is the only method to guarantee long term preservation of germplasm and simultaneously minimize the opportunity for genetic change. Cryopreservation has been successfully applied to meristems, zygotic and somatic embryos and suspension cultures of a number of different species (see Withers, 1982; 1984). Cryopreservation has been successfully applied to a broad spectrum of plant species (Withers, 1984, cites 42 species) and it can be confidently expected that successful cryopreservation can be applied to any in vitro cultured material. The chief limitation is the requirement of a liquid nitrogen refrigerator and a reliable supply of coolant.

4. Variability from in vitro culture

Variability in tissue culture has been described at all levels of the tissue culture process. Both for convenience and its implications for germplasm conservation variability in callus and suspension cultures will be treated separately from that of variation observed among plants regenerated from tissue cultures. The former is usually referred to as tissue culture instability while the latter is described as somaclonal variation. The distinction is arbitrary and the two phenomena are separated here only for clarity and to provide some historicity. Considerably more attention will be devoted to somaclonal variation because the genetic consequences of variability in tissue culture are more readily assessed in regenerated plants and their sexually or asexually propagated progeny. Moreover, in vitro storage of undifferentiated tissues in which plant regeneration is impossible or undesirable is of no consequence to the main purpose of in vitro germplasm storage.

4.1 Tissue culture instability

Since 1961 reports have appeared which document the inherent instability of tissue culture lines. Instability has been discerned at the karyotypic, morphological, biochemical and molecular levels.

4.1.1 Chromosomal instability

Bayliss (1980), following D'Amato (1978), comprehensively reviewed the reported instances of chromosomal variation in tissue cultures. The classes of variant cells included polyploid and aneuploid changes, structural changes in chromosome morphology, and mitotic aberrations including multipolar spindles, lagging chromosomes, fragments and asymetric chromatid separation. In 53 reports where the chromosomal status of callus or suspension cultures was evaluated, Bayliss (1980) records only seven where no chromosomal variants were reported. Subsequently, tissue cultures of at least two of these seven, Lolium (Ahloowalia, 1983) and Medicago sativa (Reisch and Bingham, 1981) gave rise to plants with extensive chromosomal rearrangements.

As Bayliss (1980) points out chromosomal instability in tissue cultures is clearly the expectation rather than the exception. Polyploid cells can arise from the division of endopolyploid or endoreduplicated cells in the original explant. However, the majority of polyploids or aneuploids arise during culture. For example, callus lines cloned from single cells were shown to contain diploid, tetraploid and aneuploid cells (see Baylis, 1980). These could only have arisen during culture subsequent to the single cell cloning procedure.

Structurally aberrant chromosomes can be readily observed even at the gross morphological level. Most commonly these include large and small deletions, as evidenced by loss of satellites, translocations, centric fusions, and anaphase bridges provide direct evidence of chromosome breakage. At a more refined level of observation specific structural discontinuities have been discerned by Giemsa-banding in cultures of Vicia faba (Jelaska et al., 1978), Crepis capillaris (Ashmore and Gould, 1981) and Brachycome dichromosomatica (Gould, 1982).

There is no clear cause of the enhanced frequency of chromosomal abnormalities in tissue cultures. Bayliss (1980) argues that the increase in chromosomal abnormalities results from disorganized growth of the culture. He further interprets the seeming causal relationship between the auxin 2,4-D and occurrence of chromosomal abnormalities as a result of the stimulation of disorganized growth by 2,4-D and not a direct effect of the auxin on DNA replication or mitosis.

4.1.2 Morphological changes

A phenomenon frequently encountered is the spontaneous appearance of variation amongst subclones of the one parental cell line. Chaturvedi and Mitra (1975) described two subclones of Citrus grandis callus grown under identical culture conditions. One would consistently form numerous embryoids while the other formed shoots. Selby and Collins (1976) studied 20 subclones of the cultures of each of three onion varieties. They were found to vary greatly in the following parameters: growth rate (a 3-fold difference), friability, sliminess, pigmentation (from none to deep red), and allinase activity. Clonal variation in culture morphology and growth rate has often been described (Blakely and Steward, 1964; Sievert and Hildebrandt, 1965; Davey et al., 1971; Snijman et al., 1977) as has subclonal differences in pigmentation (Davey et al., 1971; Strickland and Sunderland, 1972; Mitzukami et al., 1978). Changes to auxin habituation and cytokinin habituation (Meins, 1983) have also been frequently observed in cell culture.

4.1.3 Biochemical changes

Tissue cultures of many species produce secondary metabolites many of which have commercial potential (Staba, 1982). In several instances callus subclones have been described which vary in their ability to produce alkaloids or other secondary metabolites. For example, when 143 colonies of a Solanum laciniatum culture were examined individually for the steroidal alkaloid, solasonine, they varied from 0% to to 3% on a dry weight basis (Zenk, 1978). Similarly, Tabata et al. (1978) reported dramatic differences in nicotine production in subclones of Nicotiana rustica and N. tabacum cultures. Catharanthus roseus colonies plated from the one culture were found to differ in their contents of ajmalicine and serpentine (see Zenk, 1978). The content ranged from none to concentrations exceeding that of the parent form. Fujiwara (1982) contains many additional papers which describe variation in secondary metabolite production between callus subclones.

Isoenzyme analysis has been used to a limited extent to detect changes in tissue cultures. Isozyme pattern changes have been observed in cultures of Phaseolus vulgaris (Arnison and Boll, 1975), Hordeum hybrids (Orton, 1980) and celery (Orton, 1983a). As Orton (1983b) points out these results are equivocal because of known epigenetic and/or developmental effects on isozyme expression.

It is difficult, if not impossible, to interpret variation observed in culture in a genetic mode. Epigenetic events can equally give rise to instability in culture. Non-heritable changes, though of scientific interest, are trivial with respect to maintenance of genetic stability in in vitro germplasm conservation. However, in recent years the genetic analysis of plants regenerated from tissue culture has revealed that extensive genetic changes apparently occur during tissue culture.

4.1 Somaclonal Variation

4.2.1 Definition

It is now firmly established that frequent genetic modifications can occur during the process of tissue and cell culture. Many of these modifications are manifested as heritable mutations among the progeny of regenerated plants. This phenomenon, called somaclonal variation, can be defined as genetic variability generated during tissue culture (Larkin and Scowcroft, 1981). Identifying a variant as a somaclonal mutuant requires genetic testing of regenerants by selfing and appropriate crossing for sexually propagated species. For asexually propagated species where meiotic transmission is difficult or impossible, transmission of the trait through at least two successive clonal propagation cycles provides reasonable assurety of a true genetic base.

4.2.2 Nomenclature

The growing interest in the genetic analysis of plants derived from tissue culture has led to a need for a clear and universal nomenclature to denote the regenerated plant and its subsequent progeny. Some confusion has arisen in the literature concerning appropriate nomenclature for this purpose. Chaleff (1981) proposed the use of R for the regenerant (this symbol was transmitted to R_0 by others) and R_1, R_2 etc. for successive selfed generations. This system has also been used by others.

However, these alphanumeric notations fail to correspond to other universally adopted nomenclature in genetic and tissue culture research. The first plant following hybridization between two true-breeding parents for example is denoted as the F_1 and its selfed progeny as the F_2 generation. Segregation is first encountered in the F_2. Similarly, following mutagenesis the generations are designated M_1, M_2, etc. with segregation for a mutation first expected in the M_2. The numeric subscripts of the dihaploid DH, (de Paepe et al., 1981) and adrogenic plants, A, (Hoffmann et al., 1982) also conform to the controlled cross (F) and mutation breeding (M) notations.

To circumvent the current confusion a change in the symbol to SC (somaclone) has been proposed along with the use of numeric subscripts to correspond to the controlled cross (F) and mutation breeding (M) nomenclatures (Larkin et al., 1984). Thus the primary regenerant (somaclone) is the SC_1 plant, its progeny is the SC_2 generation, and this in turn advances in the SC_3 generation etc. A nuclear variant arising in culture would usually be heterozygous in the SC_1 plant and would be expected to segregate in the SC_2 generation.

4.2.3 Ubiquity of somaclonal variation

Table 1 is a comprehensive list of species in which somaclonal variation has been reported to date. Qualitative details for many of these cases have been summarized by Larkin and Scowcroft (1981, 1983).

Where a number of plants regenerated from cell culture have been observed, somaclonal variation appears to be the general rule. There is a range of diligence with which character variation has been observed, from great detail as in potatoes (Secor and Shepard, 1981) to little more than cursory observations as in peanuts (Bajaj et al., 1981). Many earlier reports describing plants regenerated from cell culture failed to mention phenotypic variation with any conviction. This was probably due to the entrenched assumption that clonal uniformity during in vitro propagation of plants was maintained. Several authors have made passing reference to the apparent homogeneity among regenerated plants of species such as celery (Williams and Collins, 1976), asparagus and iris (Reuther, 1977), potato (Wenzel et al., 1979) and Pennisetum purpureum (Haydu and Vasil, 1981).

An extended discussion of somaclonal variation in maize, wheat, sugarcane, tomato and potato will serve as examples to cover monocot and dicot species, diploids and polyploids, seed and asexually propagated species. The relevance of this research has largely been couched in terms of creating new variability for plant improvement where somaclonal variation is considered a potential benefit. However, this level of documentation is necessary to highlight the potential hindrance of somaclonal variation to germplasm conservation.

4.2.4 Maize

The classic genetic, cytogenetic and recently molecular research in maize has enabled a penetrating analysis of somaclonal variation. From only 77 plants regenerated from maize tissue cultures, Edallo et al. (1981) were able to identify 17 defective endosperm or seedling mutants which were phenotypically the same as classically identified mutants. In the analysis of somaclone progeny their criterion for classifying a variant as a mutant was that segregation ratios should conform to Mendelian expectations. The somaclones analyzed were derived from two different donor genotypes and progeny analysis showed that each somaclone carried an average

of one simply inherited mutation. Some plants carried more than one mutation and when more than one plant was regenerated from a callus, they often carried different mutations.

In another analysis of 51 somaclones, 8 segregated for recessive kernel mutations and one segregated for a mutation which caused premature wilting (McCoy and Phillips, 1982). Segregation for some of the mutants did not occur until the second seed generation. This suggests that the male and female flowers on the initial regenerant were genetically different, which is consistent with regenerants arising from more than one progenitor cultured cell. Alternatively, a genetic event induced during the culture phase which gave rise to somaclonal variation may persist after regeneration has occurred. Whatever the cause of such genetic chimaeras, their occurrence at least for germline cells, is rare rather than common.

Maize studies have also provided conclusive evidence that the mitochondrial genome can undergo genetic changes during cell culture. The Texas source of cytoplasmic male-sterility is sensitive to the host specific T-toxin elaborated by the causal agent of southern corn leaf blight, Drechslera maydis. Normal cytoplasm plants are male-fertile and resistant to the toxin. The toxin has been used as a selective agent in tissue cultures of T-cytoplasm maize lines (Gengenbach et al., 1977). Plants regenerated from the selected cell lines were resistant both to the toxin and to infection by the pathogen. Coincidentally, the toxin resistant plants had also reverted to male-fertility.

Separately, Brettell et al. (1980) found that among 60 plants regenerated from cms-T maize cultures grown without exposure to toxin, half were toxin-resistant and male-fertile. Umbeck and Gengenbach (1983) have confirmed this result. The conversion during tissue culture to toxin resistant, male-fertility is maternally inherited and shown to be associated with the mitochondria. A restriction endonuclease analysis of mt-DNA from a number of independently occurring male-fertile, toxin resistant somaclones revealed that in most cases the mitochondrial DNA had undergone a specific nucleotide sequence change. By comparison with the parent, 15 of 16 converted somaclones had lost a 6.6 kilobase Xho1 restriction endonuclease fragment (Gengenbach et al., 1981; Kemble et al., 1982; Umbeck and Gengenbach, 1983).

This maize cell culture research has not fulfilled the original plant breeding objective, namely, the recovery of D. maydis T-toxin resistant, male-sterile lines for use in hybrid maize programmes. However, it does demonstrate that genetic changes do occur during cell culture and that such alterations occur at the nucleotide level.

4.2.5 Wheat

It has recently become possible to regenerate plants from tissue cultures of wheat with reasonable efficiency. Many cultivars are now amenable to culture, using either immature embryos or young inflorescences as the explant (Maddock et al., 1983; Larkin et al., 1984). In the CSIRO study, the initial wheat somaclonal regenerants (SC_1 plants) displayed some phenotypic variation, but it was the analysis of their progeny which revealed the extent of the genetic changes induced during culture (Larkin et al., 1984). The analysis of progeny of 142 regenerants of a double dwarf accession, Yaqui 50E, through several consecutive seed generations showed segregation for increased height, reduced height, heading date, presence or absence of awns, glume colour, grain colour, and leaf waxiness. Segregation ratios for some,

but not all, of the qualitative characters apparently conformed to Mendelian expectations. For the quantitatively varying character, plant height, an offspring parent regression analysis gave a heritability estimate of 0.67.

A preliminary analysis of yield and yield component data from a replicated hill plot experiment involving 256 somaclonal lines derived from three cultivars indicated that somaclonal variation did affect these cultivars. Significant effects, both positive and negative relative to parental controls, were observed for grain number and total grain weight per spike, grain weight, grain yield and harvest index. In the cultivar Millewa, for example, 6 of the 100 lines significantly ($p < 0.05$) outyielded the parent control and 3 lines had significantly higher harvest index. A more extensive field trial is in progress to confirm these differences.

Biochemical analysis on seed of the progeny of some of these somaclones has also revealed extensive variation in the electrophoretic pattern of the gliadin proteins. Particular protein bands were not only lost, but "new" bands were observed and intensity changes for other fractions were also recorded. This contrasts with the striking uniformity of the gliadin patterns among seed of the parent. Some of the variant patterns breed true in subsequent generations while some continue to segregate.

An analysis of the synthesis of the secretory enzyme, α-amylase, induced by gibberellic acid (GA_3) has revealed changes in response both to GA_3 induction and to abscisic acid repression of GA_3 α-amylase induction. For these genetic variants, isoelectric focussing of both α- and β-amylases indicates that some variants have lost and/or gained specific amylase protein fractions. In others the relative concentration of particular fractions has altered relative to the parental control seed.

The results of research on somaclonal variation in wheat can be summarized as follows:

(a) Variation was manifested for both morphological and biochemical characters and for traits under simple genetic control (gliadins, grain colour) and polygenic control (height, heading date, yield).

(b) A single somaclone could be variant for a number of traits which appeared to assort independently in progeny analysis.

(c) Both heterozygous and homozygous mutants were recovered in the primary regenerant. A single somaclone could contain both states at different loci.

(d) Aneuploidy did occur but variants were present in euploid plants.

(e) Mutations affected characters for which major gene loci are known to be located on all seven homoeologous groups.

(f) Somaclonal mutants could be recessive (awns, grain colour), dominant (glume colour, awn inhibition) or codominant (gliadins).

4.2.6 Tomato

Plants regenerated from cultured leaf explants of an inbred variety carried a wide variety of mutants (Evans and Sharp, 1983). These mutants were detected in the segregating progeny of selfed somaclones. Among the progeny of 230 plants, 13 nuclear gene mutations were recovered. These mutants affected growth habit, pedicel jointedness, fruit colour, albinism, and male sterility. Three of the thirteen mutants were dominant and for one of the recessive mutants the original regenerant was homozygous for the mutant. As a control no mutants were found among more than 2,000 plants from seed of the donor parent.

In the wild species, Lycopersicon peruvianum, plants regenerated from in vitro cultures of anthers carried a surprisingly large number of mutations to new gametophytic incompatibility alleles (S-alleles) as well as S-allele reversions (Sree Ramulu, 1982). Among only 37 anther culture regenerants of one genotype, 16 had S-allele changes. From a second genotype one regenerant out of 16 showed a new S-allele specificity. This result stands in sharp contrast to numerous conventional experiments which have failed to generate any new S-alleles despite mutagenic treatments.

4.2.7 Sugarcane

Several independent studies have shown that disease resistant segregants can be recovered in sugarcane presumably as a consequence of somaclonal variation. These have been summarized by Larkin and Scowcroft (1981). They include resistance of Fiji virus disease and downy mildew (Sclerospora sacchari) (see Heinz et al., 1977) and culmicolous smut (Ustilago scitaminea) (Lui, 1981). Following earlier reports of resistance of eyespot disease (Helminthosporium sacchari) among sugarcane somaclones in Hawaii (Heinz et al., 1977), an extensive analysis has been carried out on somaclones of Australian sugarcane cultivars (Larkin and Scowcroft, 1983).

Complex callus cultures were initiated from the cultivar, Q101, (Larkin, 1982) from which plants were regenerated over a period of 6-18 months. Plants were then assayed for their reaction to the host-specific pathotoxin by measuring leachate conductivity of leaf discs exposed to a defined pathotoxin concentration (Larkin and Scowcroft, 1983). A total 480 plants were assayed. The distribution of toxin reaction was biased towards resistance and approximately 10% of the somaclones were judged resistant at least at the toxin screening level. When toxin was included in the callus phase the distribution of toxin reaction was further biased towards resistance.

A total of 85 somaclones were analyzed for the stability of their increased toxin tolerance in each of up to five subsequent vegetative generations. The majority (70%) maintained a stable reaction to the toxin while only 10% reverted towards increased sensitivity. The remaining 20% somatically segregated for resistant and susceptible plants. They may represent somaclones which were mosaic for resistant and susceptible cells. To further characterise stability, six somaclones showing vegetative stability were subjected to a second 6 month tissue culture cycle. Among 60 secondary somaclones assayed, 60% had similar or enhanced toxin tolerance relative to that of the respective primary somaclone. The remainder were more susceptible to the toxin.

The relatively high level of stability of toxin tolerance through a second culture cycle argues in favour of the notion advanced by Shepard et al. (1980) that characters can be "stacked". It seems possible that one can screen for modification of a second characteristic following a second culture cycle and expect that a workable proportion of these will retain the first characteristic recovered from selection and/or screening of somaclones derived from a first culture cycle.

4.2.8 Potato

Shepard and his colleagues (Shepard et al., 1980; Secor and Shepard, 1981; Shepard, 1981) have described the extensive variation observed among plants regenerated from cultured protoplasts of the cultivar, Russet Burbank. This old, but particularly valuable cultivar, has been effectively excluded from potato improvement programmes because it is sterile. In their initial somaclone (protaclone) experiments some 1700 plants were evaluated for overall morphological variation. In two subsequent growing seasons this population was reduced to a total of 65 clones which generally possessed acceptable vigour, vine and tuber characteristics. Statistically significant differences were found for 22 of the 35 characters and each of the 65 somaclones differed from the parent by at least one trait. The model class of 15 somaclones differed from Russet Burbank in 4 characters and one plant was distinguished from the parent by no less than 17 traits. Shepard concluded that variation from cell culture would provide enough variability to facilitate the selective improvement of Russet Burbank.

The potato somaclones were also screened for both late and early blight resistance. The parent, Russet Burbank, is highly susceptible to both of these diseases. From among more than 800 plants a considerable range of variation in reaction to late blight (Phytophthora infestans) was found. About 2% of the somaclones displayed enhanced resistance which was transmitted through subsequent tuber generations. In a similar fashion several disease resistant somaclones were recovered from a population of 500 plants screened for reistance to early blight (Alternaria solani). Five plants were initially identified as resistant on the basis of a toxin assay. Four of these subsequently displayed field resistance.

Somaclonal variation has been confirmed for several other important cultivars of potato such as Maris Bard (Thomas et al., 1982) and the widely grown European variety Bintje (Sree Ramulu et al., 1983). Significantly, somaclonal variation in potato is now being used as a breeding option in the U.K., U.S.A. and Germany.

5. Factors Influencing Somaclonal Variation

Extensive, orthogonal experiments have not yet been conducted to define the relative impact of various components and aspects of tissue culture on somaclonal variation. However, several features of tissue culture are already known to have an influence on the level of somaclonal variation in plants.

5.1 Sexual versus asexual species

One of the more important consequences of somaclonal variation is the greatly enhanced frequency of chromosomal abnormalities during tissue culture (see Section 6.1). Many of these chromosome changes will affect totipotency so that many will not be carried through to regenerated plants, i.e. morphogenesis selectivity eliminates many, though undefined, chromosomal changes. For example, Ogihara (1981) found a significant reduction in aneuploids when comparing chromosome numbers of tissue culture lines of Haworthia with plants regenerated from them. However, meiotic analysis showed that many of the regenerated plants still carried chromosome rearrangements.

There is a further barrier which eliminates chromosomal abnormalities in sexually reproducing species, namely gametogenesis and fertilization. Somaclonal mutations which cause sterility obviously will be eliminated. Thus analysis of tissue culture generated variability is likely to give different interpretations for asexually as against sexually propagated species. This is further compounded by ploidy level. In diploids such as maize (McCoy and Phillips, 1982) and tomato, gross chromosomal abnormalities among somaclone progeny are rare, whereas in polyploid species such as wheat (Karp and Maddock, 1984) and oats (McCoy et al., 1982) they are more frequent.

It is significant for germplasm conservation that asexually propagated species can be expected to display a higher frequency of somaclonal variation than those where the propagule is a seed.

5.2 Preexisting versus culture induced variation

It has been argued that variation in plants regenerated from tissue culture was preexisting in the cells of the donor explant, either as a somatic mutation or residual heterozygosity. Though neither can be ruled out as contributing some variation, evidence indicates that the great majority of variation occurs during tissue culture.

Barbier and Dulieu's (1980) extensive analysis of variation among plants regenerated from protoplasts of leaf callus showed that about 3% of regenerated plants carried a mutation at either one of two defined loci affecting chlorophyll synthesis. A genetic analysis of a sample of the altered genotypes indicated that the alleles had either reverted to wild type or had been deleted. From this analysis they could not discriminate between pre-existing and culture induced variation.

Subsequently, Barbier and Dulieu (1983), conducted an experiment in which protoplasts isolated from plants heterozygous at each of two loci affecting chlorophyll development - the a_1 and yg loci - were cultured to produce single protoplast derived colonies. Each of more than 1000 colonies were subdivided into four subcolonies from each of which a single plant was regenerated and phenotypically classified with respect to variation at the a_1 or yg loci. This analysis allowed them to conclude that 17% of the original colonies each of which had been derived from a single protoplast, had become heterogeneous at the a_1 or yg locus. While this suggests that the variation did occur during culture, it is also consistent with the notion that single strand lesions were present in the cells of the leaves from which the protoplasts were isolated. Following DNA replication during protoplast culture, mitotic segregation yielded daughter cells in which the lesion became homozygous.

A similar type of experiment in tobacco used the semi-dominant aurea mutant, Sulphur, to discriminate between pre-existing and culture induced variation (Lörz and Scowcroft, 1983). Both homozygotes and the heterozygote can be readily distinguished by phenotype as normal green (su/su), albino (Su/Su) and yellow-green (Su/su) leaves. In leaves of the Su/su heterozygote, single green or albino spots and twin spots are observed as a consequence of somatic genetic events. Protoplasts were isolated from leaves of the heterozygote and cultured to produce 2156 morphogenic colonies each of which were derived from a single protoplast. From observations on the regenerated shoots, colonies were classified as parental where all shoots were Su/su, homogeneous variant where all shoots were either Su/su or su/su, or heterogeneous variant where colonies gave rise to both Su/su and su/su, or Su/su and Su/Su shoots. The homogeneous variant colonies represent pre-existing variation, whereas the heterogeneous ones reflected genetic alteration occurring after protoplast isolation. The frequency of this latter class of colony was three times that of the homogeneous variant class, indicating that the occurrence of the somatic genetic event was significantly enhanced during tissue culture.

The genetic analyses of dihaploids produced by microscope culture in both Nicotiana sylvestris (De Paepe et al., 1981) and rape (Hoffman et al., 1982) provide convincing proof that variation occurred during the culture phase following microspore culture when spontaneous chromosome doubling occurred. In both rape and tobacco some dihaploids, which are expected to be completely homozygous, produced progeny which segregated for morphological mutants.

In an extension of this approach, Prat (1983) cultured protoplasts from a tobacco line produced by five consecutive cycles of androgenesis and chromosome doubling and subsequently selfed for two generations. A genetic analysis of plants regenerated from cultured protoplasts of this line showed that some of the regenerants were heterozygous for mutations separately affecting plant height, flowering time and albinism. Additional variation was shown for quantitatively inherited traits such as leaf and flower morphology. The pedigree of the line from which protoplasts were isolated ruled out residual heterozygosity as a source of variation and it can be concluded that the variation arose during the culture phase. Similar experiments with second cycle dihaploids in N. tabacum (Brown et al., 1983) confirm that the anther culture process generates more variabiity than can be accounted for by residual heterozygosity.

5.3 Genotype

Evidence, though limited, indicates that the genotype of the donor has a significant effect on the extent of variation generated during culture. McCoy et al. (1982) in a detailed examination of chromosomal abnormalities among plants regenerated from tissue cultures of two oat cultivars found that one cultivar consistently gave a higher frequence of chromosomal variants. Similarly in a comparison of chromosome variation in regenerants derived from protoplasts of two potato cultivars, Karp et al. (1982) found that while aneuploidy was frequent in both, the nature of the variation differed. Maris Bard regenerants had high chromosome numbers (46-92) and a wide range of aneuploidy while in the cultivar, Fortyfold, a higher proportion of plants had chromosome numbers near the normal number.

The frequency of morphological variants which appeared in the analysis of somaclonal progeny of wheat differed between cultivars (Scowcroft, unpublished). The cultivar Yaque 50E had a greater frequency than that of Millewa which in turn produced more somaclonal variants than the cultivar Warigal. In strawberry, where meristem culture is routinely used for clonal propagation, varietal differences occur in the frequency of off-type plants (Schwartz et al., 1981). Among plants derived by adventitious shoot formation from leaf explants in Begonia x hiemalis, Roest et al. (1981) found that in one variety 43% of regenerants were variant (colour, size and form of leaves and flowers) whereas for another variety only 7% were variant. Some varieties of Pelargonium are known to be inherently unstable through conventional stem cutting propagation. As expected such cultivars also produce a high frequency of abnormal plants regenerated from callus culture (Skirvin and Janick, 1976).

5.4 Explant type and culture mode

The choice of explant and the mode in which it is conserved can have a significant effect on the amount of variation generated. This may be one of the most crucial aspects of tissue culture to minimize variation for purposes of in vitro germplasm conservation.

It can be seen from Table 1 that quite diverse explants can give rise to somaclonal variants. Some explant sources and modes of producing in vitro plants, as for example adventitious shoots from petiole or leaf explants, minimize the duration of the callus phase. While such a procedure does reduce the extent of somaclonal variation, it does not obviate it. Barbier and Dulieu (1981) found somaclonal variants not only among plants regenerated from callus and cultured protoplasts but also among plants rapidly derived from adventitious shoots on leaf explants.

By inference it was considered that the extensive variation identified in potatoes by Shepard required the use of a protoplast culture system. While this is desirable from the standpoint that individual somaclones have a unique single cell origin, it is not essential for the elaboration of somaclonal variation. Thomas et al. (1982) found variation between different plants regenerated from a single callus which itself had been derived from cultured protoplasts of the cultivar Maris Bard. They also produced plants by stem embryogenesis from a different cultivar Majestic and found relatively low levels of variability which could reflect varietal differences as discussed earlier. In contrast, from a different cultivar of potato, Desirée, van Harten et al. (1981) found substantial variation for stem, leaf, flower and skin colour variants among plants regenerated by the rapid adventitious shoot technique from rachis, petiole and leaf-disc explants. In the case of plants produced from rachis and petiole explants, not previously exposed to X-rays (as had other explants), 50% of the plants were variant relative to the donor parent.

It is clear that no matter what the explant, any phase of dedifferentiated callus growth usually leads to tissue culture instability. Thus for germplasm conservation the use of a system which has a callus phase, such as adventitious shoots from leaf, petiole or stem, should be strongly avoided. The avoidance of a callus phase however does not completely obviate somaclonal variation.

There is a presupposition that variation may be avoided if plants regenerated via somatic embryogenesis. This notion rests on the assumption that the developmental constraints required by embryos will select against variant cells. This hypothesis is supported by reports of apparent uniformity among regenerants produced by somatic embryogenesis in Pennisetium spp. (Haydu and Vasil, 1981), Panicum maximum (Hanna et al., 1984) and Lolium multiflorum (Dale et al., 1981). However, these results were based on small samples or were preliminary results. For example, in Panicum maximum only 20 regenerants were evaluated for cytogenetic and morphological abnormalities. In addition, P. maximum is an apomict thus precluding the recovery of homozygous recessive progeny. More detailed analysis of L. multiflorum regenerants (Dale and Jones, 1982) revealed somaclonal variation. A more substantial isoenzyme analysis of 60 plants derived by somatic embryogenesis from a hybrid between Carica papaya and C. cauliflora showed a high degree of uniformity (Moore and Litz, 1984). Staritsky et al. (1980) also reported that coffee plants derived from somatic embryos were outwardly uniform. In other studies, the analysis of plants produced from culture by somatic embryogenesis indicate that variation can occur in coffee (Sondahl, 1982), celery (Sharp and Evans, 1982) and sugarcane (Ahloowalia and Maretzki, 1983).

It appears that the culture of isolated meristems provides the best option to minimize culture variation. However, this conclusion is difficult to substantiate because of the lack of adequate experimentation in characterising variation in meristem propagated plants. Using rapid propagation from stolon meristem tips of strawberry, Schwartz et al. (1981) evaluated some 500 plants from each of three cultivars. Variant plants were found including runnerless and female sterile types and those with compact trusses. Dwarf variants comprised 1.2% of the total population. Generally, reports on the status of plants produced by micropropagation of explanted meristems refer casually, if at all, to their relative uniformity. Defined research is needed to establish the level of variation in meristem derived plants in comparison to that found as a consequence of somatic embryogenesis and organogenesis from callus.

5.5 Culture conditions

5.5.1 Media composition

There is little evidence to suggest that the nutrient composition of the culture media has any effect on the level of tissue culture instability. Some experiments indicate that the commonly used auxin, 2-4,D, enhances the level of instability. Bayliss (1980) has reviewed the evidence and concludes that chromosome instability in culture cannot be attributed to 2-4,D per se. Rather the auxin induces dedifferentiation and it is the dedifferentiation process and its maintenance that causes chromosome instability. In their analysis of somaclonal variation of the Sulphur locus of tobacco, Lorz and Scowcroft (1983) included a nil organic nitrogen treatment to impose a stess. There was no significant effect of this treatment on somatic mutation at the Su locus.

The mutagenic activity of culture medium and particularly growth regulators have been evaluated using the Tradescantia stamen hair system (Dolézel and Novàk, 1984). This assay was developed as a sensitive system for somatic mutation resulting from ionizing radiation or chemical mutagenesis (Grant and Zura, 1982). In this system spontaneous mutant events occurred at a frequency of 0.5/100 stamens. There is a linear responsse to N-methyl-N-nitrosourea; at 500 μM NMU there is a 21 fold increase in mutant events. There was no mutagenic effect of culture media containing 5 μM of either 2,4-D, kinetin or indole acetic acid (IAA) or a combination of 5 μM 2,4-D, 10 μM kinetin and 10μM IAA. In each test the somatic mutation rate was not significantly greater than the spontaneous rate. This result provides substantial evidence that tissue culture media is not mutagenic per se.

5.5.2 Time of culture

Prolonged periods of tissue culture are known to result in an increased frequency of gross chromosomal aberrations (Bayliss, 1980; Meins, 1983). As expected the frequency of somaclonal variants among plants regenerated from tissue culture also increases with prolongation of the culture period.

A common observation is that tissue cultures lose totipotency with time in culture. The most logical explanation for this is the loss or mutation of genes which are essential for regeneration. Indeed such mutants which block specific steps in the regeneration process are being recovered from tissue culture derived plants.

In oats the frequency of cytogenetically abnormal plants increased dramatically with increased time in culture (McCoy et al., 1982). Abnormalities included chromosome breakage, chromosome loss, interchanges and aneuploids. Plants were regenerated from cultures of two oat cultivars, Lodi and Tippecanoe, after 4, 8, 12, 16 and 20 months in culture. Among Tippecanoe regenerants the frequency of observable chromosome aberrations rose from 49% after 4 months culture to 88% after 20 months. For Lodi over the same period the change was 12% to 48%. Similar observations have been made among plants regenerated from tissue cultures of different ages of triticale (Armstrong et al., 1983) and wheat (Karp and Maddock, 1984). In sugarcane, plants regenerated from tissue culture at various intervals from 6 months to 18 months after culture initiation were assayed for tolerance to the toxin produced by Helminthosporium sacchari which causes eyespot disease (Larkin and Scowcroft, 1983). The frequency of toxin tolerant plants predictably increased with duration of tissue culture.

A similar effect of culture duration was also found for tobacco plants regenerated from leaft protoplasts of the sulphur mutant (Lorz and Scowcroft, 1983). Doubling the time in culture from 3 to 6 months more than doubled the frequency of somaclonal mutations at the Su locus. However, more extensive experiments by Barbier and Dulieu (1983) gave a result which is at variance with the consensus that culture duration increases the level of variation. Protoplasts were isolated from leaves heterozygous for two loci affecting chloroplast development, and cultured. Twenty five colonies each were subcloned into 4 sectors after each of 3 successive culture periods. The analysis of plants regenerated from the subclones enabled Barbier and Dulieu (1983) to conclude that variation occurred only during the first culture period. Additional variants at either of the two loci were not observed in the second or third culture phases.

Despite this last result, for the purposes of germplasm conservation, the overwhelming weight of evidence argues that variation will be minimised if the duration of callus culture is kept as short as possible.

6. Origin of Tissue Culture Instability and Somaclonal Variation

An understanding of the mechanisms which give rise to tissue culture instability and somaclonal variation is necessary for several practical reasons. First, where somaclonal variation is to be utilized to generate variability for plant improvement it could be desirable to enhance its occurrence. Equally important where uniformity of tissue culture plants is essential, as in rapid micropropagation or for *in vitro* germplasm conservation, it is necessary to be able to control the mechanisms which generate variation.

Apart from chromosomal aberrations there is insufficient evidence to support, let alone favour, any one of several possible mechanisms to account for somaclonal variation. Several of these possibilities will be considered primarily because they reflect the direction of current research.

6.1 Chromosomal aberrations

It has been known for some time that chromosomal abnormalities occur at a high frequency in tissue cultures (see Section 4.1.1). Among regenerated plants and their progeny the incidence of gross chromosomal aberrations is greatly reduced relative to cells in tissue cultures. In the analysis of regenerants, chromosomal aberrations which preclude morphogenesis will not be observed. Where somaclonal progeny are analysed those aberrations which cause sterility will also be eliminated. As pointed out in Section 5.1 the requirement of morphogenesis and fertility is a selection process which eliminates many chromosomal abnormalities and strongly favours euploidy.

In an extensive meiotic analysis of plants regenerated from two cultivars of oats, McCoy *et al.* (1982) found that the common abnormality was breakage and loss of chromosomal segments. Among more than 300 regenerants for each of two cultivars approximately 20% of the plants carried one or more interchanges and/or breakage events as evidenced by the occurrence of heteromorphic pairs at diakinesis. Only about 6% of regenerated plants were aneuploid. This analysis also indicated that the frequency of chromosomal rearrangement increased with time in culture and that there was a cultivar effect on the frequency of aberrations among the regenerants.

In wheat, karyotype abnormalities have also been identified in regenerated plants (Karp and Maddock, 1984). Among 192 plants regenerated from four different wheat cultivars, 29% were aneuploid. A meiotic analysis of regenerants showed that interchanges were particularly common. In our own cytogenetic analysis of wheat somaclones we have found that aneuploidy is infrequent, as would be expected since our analysis was done on progeny of fertile somaclones (unpublished data). However, among these progeny we have observed both ring and open quadrivalents which presumably result from translocation events. These interchanges are not necessarily associated with morphological mutants.

A causal relationship between gross chromosomal aberration and genetic changes in somaclones is incompletely resolved. Orton (1983b) sought a correlation between chromosome loss and genetic changes at the phosphoglucomutase (PGM) locus in celery callus cultures and regenerated plants. A causal relationship could not be established despite the fact that chromosome loss and instability at the Pgm-2 locus were observed. The two manifestations of somaclonal variation appeared to be independent. However, since chromosome rearrangements can only be seen cytologically if they are a certain size or type, it may only be our discontinuous powers of resolution of the variation which make chromosome rearrangements and single gene mutations appear to result from separate mechanisms. Specific gene effects can result from chromosomal interchanges because of gene inactivation, position effects and altered developmental timing. Chromosomal abnormalities are part of the spectrum of somaclonal variation and they, as well as other classes of somaclonal variants, may have a more fundamental biological basis.

6.2 DNA Amplification

Plant genomes are large with haploid genome sizes in the range of 10^9-10^{10} nucleotide pairs. Though variable from one species to another, about 60% of the genome of a "typical" plant is composed of repeated DNA. Most of the repeated sequence DNA is low or moderately repetitive DNA with a reiteration frequency per sequence of less than 10^5. Some highly repetitive DNA (10^5-10^6 copies) also occurs in most plants and can comprise up to 20% of the total DNA as in maize (Sorensen, 1984). Repetitive DNA is of two basic types. The repeat units can be clustered as arrays of tandem repeats with specific genome location, or the repeat family can be interspersed throughout the genome, usually in different repeat unit permutations.

One class of genes which is highly repeated in plants is that which codes for ribosomal RNA (rDNA). The 26, 18 and 5S rDNA genes in plants, as in animals, are transcribed as a single unit. Typically this major transcriptional unit ranges from 7.8 to 12.7 kilobase pairs. The reiteration frequency is highly variable from species to species. The number of copies can be as few as 1300 per nucleus or as many as 31,900. Sorensen (1984) reflects a consensus that the reiteration frequency of rDNA in higher plants is dynamic.

Both qualitative and quantitative changes in DNA of tissue cultures and regenerated plants have been documented. A kinetic analysis of DNA extracted from pith callus cultures (Durante *et al.*, 1983) indicated that there was differential replication of both GC and AT rich sequences during the early phases of dedifferentiation. They interpret these results as a primary step in the cascade leading to dedifferentiation.

In an extensive analysis of dihaploid plants of *N. sylvestris* derived by consecutive androgenesis. De Paepe et al. (1982) identified both heritable quantitative and qualitative changes in the nuclear DNA. An increasing proportion of a 1.703 satellite signalled an increase in GC rich sequences or alternatively an increase in the degree of methylation. Thermal denaturation also indicated an increase in AT rich sequences. An analysis of zero-time binding sequences following 51 nuclease treatment showed that there was a progressive increase of inverted repeat sequences in consecutive dihaploid plants. This was particularly prevalent in DNA sequences 200-400 nucleotides long.

It is reasonable to conclude therefore, that DNA sequence amplification, or deamplification, could be one of the mechanisms responsible for somaclonal variation. Such amplification could lead either to increased synthesis of a specific gene product or to perturbations in developmental timing of gene activity if the repeated sequences function in new chromosomal locations.

6.3 Transportable Elements

The penetrating genetic analysis of unstable mutations in maize enabled Barbara McClintock to postulate the existence of genetic elements which transpose from one location in the genome to another (McClintock, 1951). Transposition occurs in both somatic and germline cells. Recent molecular and genetic analysis has shown the occurrence of transposable elements in eukaryotes to be the rule rather than the exception (Shapiro and Cordell, 1982). In plants, mutants at the shrunken, waxy and alcohol dehydrogenase loci of maize and the chalcone synthase locus of *Antirrhinum* have been found which contain identifiable transposable elements (see Flavell, 1984). Mutant phenotypes can result either from insertion of the element into the structural gene or into the regulator gene sequences.

By virtue of their movement, transposable elements can inactivate structural genes, alter gene regulation, possibly reactivate silenced genes, and can generate duplications and deficiencies. Though not understood, genomic and developmental shock can induce the transposition of mobile elements. The breakage-fusion-bridge cycle used by McClintock (1951) to generate unstable patterns of mitotic segregation for a chromosome segment is a classical example. In Drosophila strains which have been destabilized by hybrid dysgenesis, "transposition bursts" occur in which many transposition events occur (Gerasimova *et al.*, 1984). The transposition bursts were interpreted as occurring in pre-meiotic cells of the germ line.

Concrete evidence has yet to be presented that transposition events are a cause of somaclonal variation in plants. Several examples however, do provide circumstantial evidence of events which are analogous to transposition. In Shepard's analysis of somaclonal variation in potatoes (see Section 4.2.8), somaclones were identified which simultaneously differed from the parent cultivar in up to 17 characters. Among the 65 somaclones, each one was distinguishable from the parent by an average of 7 characters. Such coincident variation could be explained by transposition burst. Similarly we have observed a concurrence of variation among wheat somaclones (Larkin *et al.*, 1984). Several somaclone families were simultaneously variant for many characters. For example, progeny derived from a single somaclone segregated for height, maturity date, presence or absence of awns, grain colour and had altered gliadin protein fractionation patterns.

Simultaneous independent mutants have also been reported in tobacco and lucerne. Chaleff and Keil (1981) utilised selection in cell culture to produce several independent herbicide (picloram) resistant plants. The resistance was inherited as either a dominant or semi-dominant gene. Subsequently they found that three of the five picloram resistant genotypes, unlike the parental plants, were also resistant to hydroxyurea. This resistance was inherited as a single dominant mutation and surprisingly, in two of the three cases, assorted independently from picloram resistance. In lucerne, Reisch and Bingham (1981) observed a far greater amount of variation among plants regenerated from cell lines previously subjected to selection for resistance to ethionine than among plants regenerated from unselected callus. The variation was reflected in leaf and growth habit, plant height, shoot length, number of primary branches and field plot yield. The authors attributed the variation to impaired DNA replication and/or repair as a consequence of ethionine induced transethylation of nucleic acids. It is equally likely, in both this and the tobacco cases, that plants selected for a particular mutation would also carry mutants at other loci if multiple transposition had occurred.

Unstable mutants have also been reported among plants regenerated from cell and tissue culture. In section 5.2 the results of the effect of cell culture on the allelic state of the Su locus in tobacco were discussed. Plants regenerated and grown to maturity carried other independent mutants which affected leaf, flower and whole plant morphology and in many plants meiotic segregation was aberrant. One type of somaclonal mutant, for which there were two independent observations among 120 regenerants, affected the frequency of both single and twin spots on the leaves of Su/su heterozygotes. The frequency of leaf spotting relative to non-culture derived plants was elevated up to 500 fold. This "Su mutator" somaclonal variant is inherited as a dominant mutant which appears to be lethal in the homozygous condition. The mutator is unstable and can be spontaneously lost either in somatic tissue or during meiosis. In lucerne, Groose and Bingham (1984) recovered a white flowered somaclone from a purple-flowered parent. Chromosome loss did not account for this variant. Following a second culture cycle of the white variant, some regenerants had reverted to purple flower type. Both the original mutant and the revertant are heritable. Reversion rate among culture derived plants was as high as 50% and the instability of the mutant allele was itself heritable.

6.4 Other Mechanisms

Other possible mechanisms which could give rise to somaclonal variation have been enumerated by Larkin and Scowcroft (1981), Scowcroft and Larkin (1983) and Orton (1983b). They include somatic rearrangement of genes particularly those in multigene families, somatic crossing over,sister chromatid exchange, altered nucleotide methylation patterns, perturbation of DNA replication by altered nucleotide pools, and silencing or activation of genes by mutations in associated non-coding regions. Many of the answers to the causes of tissue culture instability and somaclonal variation will result from molecular analysis. Somaclonal mutants are currently being isolated at loci for which a DNA probe is available. This will enable cloning of the somaclonal mutant gene and its analysis to determine the cause of altered expression. A non-genetic cause of variation which is particularly relevant to asexually propagated crops is virus elimination during tissue culture.

6.5 Virus elimination

Tissue culture and especially meristem culture of asexually propagated species is an established method to eliminate viruses from plants. Examples include potato, cassava, strawberry, rhubarb, grape and several floricultural species. As well as known viruses, those which give no obvious symptoms (cryptic viruses), may be eliminated by tissue culture. The consequences of virus elimination usually lead to improved agronomic performance which could be interpreted as beneficial somaclonal variation. This is particularly problematic for asexually propagated species where genetic analysis is not possible.

7. Impact of Culture Instability and Somaclonal Variation

The primary mandate of the IBPGR is the conservation of the genetic diversity of economic plants. However the efficacy of this effort will be judged largely on the exchange and utilization of conserved genetic resources. For a significant number of species adequate conservation can only be achieved by *in vitro* techniques. Under *in vitro* conditions genetic modifications occur at a relatively high rate. There are both benefits and disbenefits of this for germplasm conservation, exchange and utilization.

7.1 In vitro germplasm conservation

The impact of tissue culture instability and somaclonal variation on genetic modification relies almost exclusively on its analysis in seed propagated species. The inability to conduct genetic analysis for most asexually propagated species makes monitoring of genetically important tissue culture instability almost impossible. It can only be concluded therefore that similar principles and consequences gleaned from studies of somaclonal variation in sexual species also apply to asexually propagated species. For these species it can be further assumed that higher levels of phenotypic variability might be observed in plants regenerated from *in vitro* conserved tissues because deletions, aneuploidy etc. which can be more readily tolerated will expose previously existing recessive alleles. In sexually propagated species such gross genetic modifications are usually eliminated during gametogenesis and/or fertilization. It can be further assumed that many of the recessive genetic changes which occur during tissue culture of asexually propagated species will have no phenotypic expression, i.e. they remain hidden because of heterozygosity. Thus, as a consequence of *in vitro* conservation, germplasm resources of asexually propagated species are likely to contain relatively more genetic variation than in species where seed is the conserved propagule.

Where there is an option to conserve genetic resources as seed, this must be considered the preferred option. For highly heterozygous and often polyploid species which have a storable seed, e.g. cassava, both seed and *in vitro* methods are the only way to conserve unique, coadapted gene complexes in highly heterozygous species. In cassava such complexes probably exist in many cultivars. Where specific genes are known for particular traits, e.g. disease resistance and stress tolerance, such resources are best conserved by seed.

Tissue culture instability and somaclonal variation make the use of minimal growth techniques for germplasm conservation potentially hazardous for two main reasons. First, the imposition of an environmental stress on cultures could induce mechanisms which cause genetic modification such as DNA sequence transposition, somatic recombination or sequence amplificaton. Factual demonstration that this does occur in culture derived plants is not yet available, but on the basis of research in other eukaryotes, such a consequence is fully expected. Second, minimal growth conditions impose definite selection pressures. It is established that in vitro selection is a very effective means to generate mutants in plants. The use of minimal growth conditions over long periods for germplasm conservation can be expected to lead to directional genetic change.

Cryopreservation should obviate the constraints of minimal growth methods because the presumed biological processes causing instability are arrested at ultra-low temperatures. From studies on cryopreservation of seeds, cryopreservation appears not to affect either chromosome integrity or mutation rate. Similar experiments have not been done with cryopreserved tissue cultures or plant organs.

Dedifferentiation of plant tissues either induces or predisposes cells to genetic modification. In vitro storage methods should avoid using dedifferentiated tissues such as callus or suspension cultures for germplasm conservation. The genetic integrity of differentiated plant structures is also at risk if they have been derived from callus or suspension cultures. This obviously includes plants regenerated from cultured protoplasts. Thus the genetic integrity of somatic embryos relative to their donor will depend on the nature of tissue from which they were derived. For example, the production of somatic embryos directly from explanted stems, petioles or leaves are less likely to carry genetic modifications than somatic embryos derived from callus or cultured protoplasts each of which involved a dedifferentiated callus phase prior to regeneration.

Meristems, amplified by micropropagation, appear to be at least subject to culture induced genetic modification. As for somatic embryogenesis, callus formation should be avoided during the meristem micropropagation process in order to minimise genetic instability. Generally, for in vitro germplasm conservation, meristems are preferred over somatic embryos. Despite the fact that callus or suspension cultures are easier to cryopreserve, germplasm storage using this type of material should be avoided at all times.

7.2 In vitro germplasm exchange

The obvious effect of genetic instability on exchange of in vitro conserved germplasm is the finite chance that a recipient of germplasm may not receive a cultivar which is true to type. This is a particular disbenefit of conservation using minimal growth conditions. A possible solution is that exchange should involve more than one tissue culture propagule each of which have been derived from different explants.

One of the major benefits of using tissue culture, particularly meristems, for exchange derives from the elimination of disease pathogens. The sterile conditions under which in vitro derived propagules are raised largely excludes the transfer of bacterial and fungal diseases. Micropropagation is also very effective for eliminating disease viruses, particularly if the donor explant has been subjected to chemo- and/or thermotherapy. It has not been established whether tissue culture will eliminate mycoplasmas from asexual propagules. As pointed out in Section 6.5 those concerned with the exchange of in vitro consumed germplasm may wrongly interpret phenotypic changes resulting from virus elimination as genetic instability.

It is now possible to develop nucleic acid probes against virtually any plant virus. These molecular probes can be used to detect the presence of virus nuclei acids even in the absence of symptoms. These molecular probes enable early, rapid and non-destructive screening of small tissue samples. Typically, meristems could be non-destructively screened prior to shipment and classified as specific virus free within a few days.

7.3 Germplasm utilization

The focus of the report is to evaluate the hazards of tissue culture instability and somaclonal variation on in vitro germplasm conservation. However, the consequences of chromosomal instability in tissue culture is now considered to have benefit for more effective germplasm utilization.

Wild relatives of the major crop species are recognized as actual and potential contributors of genes for plant improvement. In crops such as tomato, the incorporation of exotic genes into commercial cultivars has been impressive and in wheat several genes for rust resistance have been transferred from wild relatives. A new option for achieving alien gene introgression has emerged from the observation that chromosome rearrangements occur in plants regenerated from tissue culture (see Section 6.1). The occurrence of chromosomal rearrangements during the tissue culture of interspecific hybrids and synthetic crop species, such as Triticale, is particularly compelling. Armstrong et al. (1983) found that karyotypes of Triticale were quite unstable during culture. Aneuploidy was frequent and among 51 plants regenerated from 6 month old cultures more than half the plants possessed altered chromosomes. These included telocentrics, acrocentrics, deletions and euchromatic additions. C-banding enabled identification of rye-wheat recombinant chromosomes.

In ryegress, 4000 plants have been derived from callus initiated from a single immature triploid embryo (Ahloowalia, 1983). The triploid embryo was from a cross between diploid Lolium multiflorum and tetraploid L. perenne. The regenerated plants showed wide variation in leaf shape and size, floral development, vigour and survival. Interestingly some plants displayed novel phenotypes. Cytological analysis of the triploids (2n = 21) and near triploids (2n = 19,20) revealed frequent multivalent formation which is characteristic of reciprocal translocations. In some plants penta- and hexavalent associations were observed which signifies that more than one reciprocal translocation had occurred during culture.

Larkin and Scowcroft (1981), McCoy and Phillips (1982) and Ahloowalia (1983) recognized that the enhanced level of chromosome recombination in tissue culture might be employed to effect alien gene transfer. In crops such as wheat, wide crossing programmes have produced alien chromosome addition lines following hybridization with relatives such as Aegilops, Agropyron, Elymus, Haynaldia and Hordeum. These alien species carry genes for resistance to wheat disease pathogens and tolerance to environmental stress such as heat, salinity and metal toxicity. Research is now being initiated to utilize tissue culture in an attempt to enhance the rate of transfer of specific alien genes from these wheat alien addition lines. This could emerge as one of the most salutary consequences of somaclonal variation for germplasm utilization.

8. Conclusions

8.1 In vitro culture methods are essential to conserve germplasm resources for species where it is impractical, difficult or impossible to conserve seed.

8.2 Tissue culture instability and somaclonal variation is ubiquitous. This variation has possible benefits as an adjunct to plant improvement but is a serious disadvantage for in vitro germplasm conservation and exchange.

8.3 Variation occurs at all tissue culture levels. Genetic instability and somaclonal variation has been recorded in callus, liquid suspensions, cultured protoplasts, anther culture, adventitious shoots, somatic embryos, in plants regenerated from tissue cultures and among seed and vegetative propagules of regenerants.

8.4 As a consequence of instability there is variation in ploidy levels and chromosome structure, morphological and biochemical traits and changes at the nucleotide level have been observed for both nuclear and cytoplasmic genomes.

8.5 Genetic analyses show that somaclonal mutants can be under simple or polygenic inheritance, may be recessive or dominant and mutants can be unstable.

8.6 Somaclonal variation is more likely to be enhanced, a) in asexually propagated species relative to seed propagated species; b) if callus formation is a significant phase in the regeneration cycle; c) with increased duration of culture; d) in certain genotypes; e) from specific explants.

8.7 In vitro plant culture systems may be ranked for instability (low to high) as meristem multiplication, adventitious shoots, somatic embryogenesis, organogenesis from callus and cultured protoplasts.

8.8 Instability and somaclonal variation can arise from but may not be causally related to chromosomal aberrations. DNA amplification or diminution and DNA sequence transposition are included in mechanisms which could cause somaclonal variation. The effect of virus elimination on phenotype may be wrongly interpreted as somaclonal variation. Tissue culture media and growth regulators appear not to be mutagenic.

8.9 *In vitro* germplasm storage currently uses slow growth techniques rather than cryopreservation. Minimal growth conditions put germplasm integrity at serious risk because of directional genetic change in response to selection, and somaclonal variation is possibly enhanced, particularly where regeneration is adventitious. Further work is necessary.1/

8.10 On current knowledge cryopreservation of meristems short tips provides the greatest security against genetic instability during in vitro germplasm conservation.

1/ The IBPGR *In Vitro* Committee is preparing a special study.

9. Table 1. Species displaying somaclonal variation

The explant refers to the plant organ(s) from which the cell lines or *in vitro* culture was initiated. The regeneration mode describes the culture conditions from which plants were regenerated. A single or two word description is admittedly inadequate. Callus, for example can be disorganised as in the case of tobacco, or have organised meristems as is found with most of the monocots. The adventitiuos shoot mode may involve a more or less discernible callus phase. Androgenesis implies that the somaclones evaluated were either haploids or dihaploids. SC_1, SC_2 - see text, section 4.2.1; nr - not reported.

Species	Explant	Regeneration Mode	Variant Character in SC_1 or SC_n Plants	Transmission Asexual	Transmission Sexual	Reference
A. Monocots						
Allium sativa	shoot tips, leaf base	callus	bulb size and shape, clove no., aerial bulbil germination	+		Novak (1980)
Avena sativa	(a) immature embryo, apical meristem	callus	plant ht, heading date, leaf striping, twin culms, awns		+	Cummings *et al*. (1976)
	(b) immature embryo	callus	cytogenetic abnormalities e.g. chromosome loss, interchanges, trisomy, monosomy			ſcCoy *et al*. (1982)
Haworthia	flower bud	callus	chromsome no., and behaviour, vigour, leaf shape, leaf colour, stomata no., esterase isozymes	nr		)gihara (1981)
Hordeum spp.	(a) immature embryo	callus	plant ht, tillering, albinism			)eambrogio & Dale (1980)
	(b) anthers	callus	cytological abnormalities	nr		ſix *et al*. (1978)
	(c) immature ovaries	callus	growth habit and rate, size, head morphology, auricles	nr		)rton (1980)
Lolium spp. hybrid	immature 3N embryo	callus	leaf size, flower, vigour, survival			ıhloowalia (1983)
Oryza sativa	(a) seeedling	callus	tiller no., fertile tiller no., panicle length, seed fertility	nr		ſenke *et al*. (1978)
	(b) dihaploid seed	callus	chlorophyll content, seed fertility, plant ht, flowering date, grain no., kernel wt.		+	Oono (1981)

Table 1. (contd)

Species	Explant	Regeneration Mode	Variant Character in SC_1 or SC_n Plants	Transmission Asexual	Transmission Sexual	Reference
	(c) dihaploid immature embryos	callus	height, seed no. and size, panicle size, tiller no., yield, protein	+		Schaeffer (1981) Schaeffer et al. (1984)
	(d) mature embryo	callus	heading date, culm length, sterility		+	Fukai (1983)
	(e) seeds	callus	ht, grain wt, chlorophyll mutants		+	Sun et al., (1983)
Saccharum officinarum	(a) parenchyma, inflorescence, young leaf	callus	eyespot, Fiji virus, downy mildew diseases	+		Heinz et al. (1977)
	(b) meristems, inflorescence, young leaf	callus	auricle length, dewlap shape, top leaf attitude, chromosome no., esterase isozymes, cane yield, sugar yield, stalk no., smut resistance	+		Liu (1981)
	(c) leaf base	callus	eyespot disease, leaf scald	+		Larkin and Scowcroft (1983)
Sorghum bicolor	immature embryos, seedlings	callus	fertility, leaf morphology, growth habit	nr		Gamborg et al. (1977)
Triticum aestivum	(a) immature embryo	callus	plant ht, stem thickness, leaf size, spike shape, pollen fertility	nr		Ahloowahlia (1982)
	(b) immature embryo	callus	plant and head morphology, awns, chlorophyll deficiency, gliadins, amylase, grain wt, yield		+	Larkin et al., (1984)
	(c) immature embryo	callus	chromosome aberrations		+	Karp and Maddock (1984)
Zea mays	(a) immature embryo	callus	abphyll syndrome, pollen fertility	nr		Green (1977)
	(b) Immature embryo	callus	Dreschslera maydis race T toxin resistance, male fertility, mtDNA sequence rearrangement		+	Brettell et al. (1980); Kemble et al. (1981); Umbeck and Gengenbach (1983)
	(c) immature embryo	callus	endosperm and seedling mutants		+	Edallo et al. (1981)
	(d) immature embryo	callus	defective kernel, wilting		+	McCoy and Phillips (1982)

Table 1. (contd.)

Species	Explant	Regeneration Mode	Variant Character in SC_1 or SC_n Plants	Transmission Asexual	Transmission Sexual	Reference
	(e) immature embryo	callus	dwarf, leaf morphology chloroplast mutants		+	Beckert et al., (1983)
B. Dicots						
Ananas comosus	syncarp, slip, crown, axillary bud	callus	spine & leaf colour, waxiness, foliage density, leaf width & spines	nr		Wakasa (1979)
Apium graveolens	petioles	callus	phosphoglucomutase isozyme	nr		Orton (1983b)
Arachis hypogaea	anther	callus	chromosome no.	nr		Bajaj et al. (1981)
Begonia x hiemalis	leaf	adv. shoot	colour, size, form of flowers & leaves	+		Roest et al. (1981)
Brassica napus	(a) anther	androgenesis	flowering time, glucosinolates, growth habit		+	Wenzel (1980); Hoffman et al. (1982)
	(b) androgenic embryo	callus	resistance/tolerance to Phoma lingam		+	Sacristan (1982)
B. oleracea	root crown meristem	adv. shoots	waxiness, stem branching precocious flower formation	+		Grout and Crisp (1980)
Chrysanthemum	(a) nodes, shoot tip	adv. shoot, callus	flower colour, flower induction temperature	nr		Jung-Heiliger and Horn (1980)
	(b) shoot tip	callus	shoot and leaf morphology	nr		Sutter and Langhams (1981)
Fragaria ananassa	stolon tips	adv. shoots	vigour, runnering, chlorosis, compact habit, yield	+		Schwartz et al. (1981)
Lactuca sativa	(a) cotyledons	callus	leaf wt, length, width, flatness & colour, bud no.		+	Sibi (1976)
	(b) leaf	protoplasts	cotyledon colour, leaf morphology	nr		Engler and Grogan (1983)
Lycopersicon esculentum	(a) cotyledon	callus	leaf morphology, branching habit		+	Sibi (1981)
	(b) leaf	callus	fruit colour, pedicel, male fertility, growth, chlorophyll		+	Evans and Sharp (1983)
L. peruvianum	anther	callus	self-incompatibility	nr		Sree Ramulu (1982)

Table 1. (contd.)

Species	Explant	Regeneration Mode	Variant Character in SC_1 or SC_n Plants	Transmission Asexual	Transmission Sexual	Reference
Medicago sativa	immature ovaries	suspension/ callus	multifoliate leaves, elongated petioles, growth habit, primary branch no., plant ht, dry matter yield		+	Reisch and Bingham (1981)
Nicotiana	(a) anther	androgenesis	crumpled leaf		+	De Paepe *et al*. (1981)
	(b) anther	androgenesis	amplification AT & GC rich regions	nr		De Paepe *et al*. (1983)
	(c) leaf	protoplast	ht, flowering time, albino		+	Prat (1983)
N. glauca	pith	callus	amplification AT & GC rich regions	nr		Durante *et al*. (1983)
N. tabacum	(a) anther	androgenesis	plant ht, stem diameter, leaf size, yield		+	Oinuma and Yoshida (1974)
	(b) anther	androgenesis	yield, grade index, flowering time, plant ht, leaf number, length & width, alkaloids, reducing sugars		+	Burk and Matzinger (1976)
	(c) leaf	protoplast callus, adv. shoots	2 specific leaf colour loci, leaf shape & size		+	Barbier and Dulieu (1981)
	(d) leaf	protoplast	flower & plant morphology, mutator allele		+	Lorz and Scowcroft (1983)
	(e) dihaploid leaf	protoplast	ht, flowering time, leaf morphology		+	Prat (1983)
Pelargonium	stem	callus	plant, leaf & flower morphology, essential oils, fascination, anthocyanin pigmentation, pubescence	+		Skirvin and Janick (1976)
Solanum tuberosum	(a) leaf	protoplast	tuber shape, yield, maturity date, photoperiod, plant morphology, early and late blight resistance, numerous field traits	+		Shepard *et al*. (1980); Secor and Shepard (1981)
	(b) leaf	protoplast	leaf colour & morphology vigour, ht, anthocyanin pigment	+		Thomas *et al*. (1982)

Species	Explant	Regeneration Mode	Variant Character in SC_1 or SC_n Plants	Transmission Asexual	Transmission Sexual	Reference
	(c) leaf	adv. shoots	stem, leaf, flower morphology, skin colour	+		van Harten *et al*. (1981)
	(d) shoots	protoplasts	growth, leaf and stem morphology		nr	Sree Ramulu *et al*. (1983)

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