Chapter 9

Variant Selection

9.1. INTRODUCTION

For a considerable time, the frequently observed variability in plant populations regenerated from tissue cultures was ignored, and organogenic and embryogenic differentiation in callus and suspension cultures was regarded as a potential method for rapid clonal multiplication of plant species. This being the fastest in vitro method for asexual multiplication of several plant species (Chapter 16), efforts are continuing to develop methods for large scale production of somatic embryos, in bioreactors, which can be converted into synthetic seeds for mechanized planting in the field (Chapter 6). However, a major obstacle in realization of this goal is the inherent genetic instability of cultured cells, endangering the clonal nature of the plants produced by this method, and so far there is no effective method known to control this phenomenon. The extent of variation in tissue culture raised plants is often so great that tissue culture is proving to be a rich source of genetic variability, suitable for crop improvement (Sections 9.3, 9.7).

The variations observed in tissue cultures may be due to physiological changes induced by the culture conditions. Such variations are temporary and disappear when the culture conditions are removed. However, sometimes the altered phenotype may persist over a longer period, well after the inductive conditions are withdrawn and may be passed from one cell generation to another. Such variations, which are also ultimately reversible, and are not sexually transmitted, are caused by epigenetic changes. The cultured cells also exhibit genetic variation (Table 9.1). Thus, the plants regenerated from tissue cultures show a range of variability, ranging from temporary changes in the phenotype to sexually heritable mutations. Although tissue culture induces the most obvious changes in the nuclear genome of plants, the genetic make-up of the cytoplasmic organelles can also be altered (Section 9.5).

The first formal report of morphological variation, observed in plants regenerated from sugarcane tissue cultures, was published in 1971, by Heinz and Mee. Since then several useful variants of sugarcane resistant to fungal and viral diseases have been isolated in Fiji, Hawaii and Tai-

TABLE 9.1

Criteria for the classification of tissue culture variation as genetic or epigenetic

Feature	Genetic	Epigenetic
1. Frequency of occurrence	Low; 10^{-5}–10^{-7} per cell generation	High; 10^{-3} per cell generation
2. Nature of change	Random	Directed
3. Stability of change in somatic lineages	Usually stable	Stable; but reversal can occur at high rates
4. Sexual transmission of the change	Yes	No

Based on Stafford (1991).

wan. This was followed by similar observations with other crops. Larkin and Scowcroft (1981) reviewed the scattered literature on the occurrence of variability in tissue cultures and suggested that this variation among regenerated plants could be useful for the development of new cultivars. This paper proved to be a major thrust in the exploitation of tissue culture-generated variability in crop improvement. To date, at least ten new varieties of crop plants, based on the utilization of tissue culture derived variability, have been released (Table 9.2).

9.2. TERMINOLOGY

Genetic variants selected through tissue culture have been variously referred to as calliclones (from callus cultures; Skirvin, 1978), protoclones (from protoplast cultures; Shepard et al., 1980) and phenoclones (Sibi, 1976). Larkin and Scowcroft (1981) proposed a general term 'Somaclonal variation' to describe the genetic variation in plants regenerated from any form of cell culture. Accordingly, the plants derived from cell and tissue cultures are termed 'somaclones', and the plants displaying variation as 'somaclonal variants'. Evans et al. (1984) suggested that the plants regenerated from cell cultures of gametic origin be termed 'gametoclones' to distinguish them from somatic cell derived regenerants.. Some examples of gametoclonal variation are described in Section 7.9.2. In this chapter some prominent examples of somaclonal variants showing agronomic promise are described, and the possible causes and origin of these variations are discussed.

The plants regenerated from tissue cultures are designated as R_0 generation and their successive sexual generations as R_1, R_2, and so on.

TABLE 9.2

Crop species in which new varieties have been developed from somaclones

Crop	Country and institution	New trait	Reference
Geranium	Dept. Horticulture, Pardue Univ., USA	Vigour and attractive flowers	Skirvin and Janick (1976b)
Sweet potato	North Carolina Research Service, USA	Colour, shape and baking quality of roots	Moyer and Collins (1983)
Sugarcane	Sugarcane Research Centre, Fiji	Yield and disease resistance	Cited in Daub (1986)
Maize	Molecular Genetics, USA	Tryptophan content	Anonymous (1987)
Tomato	DNA Plant Technology of New Jersey, USA	Dry matter content	Evans (1989)
		Disease resistance	Evans (1989)
Rice	Plantech Research Institute, Japan	Yield	Anonymous (1989)
	Univ. Agricultural Sciences, Godollo, Hungary	Disease resistance	Heszky and Simon-Kiss (1992)
Celery	DNA Plant Technology of New Jersey, USA	Processing, yield efficiency	Orton and Romig (1990)
Brown mustard	ICAR, New Delhi India	Yield	Katiyar and Chopra (1995)

Based on Semal and Lepoivre (1990).

9.3. SELECTED EXAMPLES OF SOMACLONAL VARIANTS

To be of agronomic use, a somaclonal variant must fulfil certain basic requirements (Gunn and Day, 1986): (i) it must involve useful character(s); (ii) it must be superior to the parents in the character(s) in which improvement is sought; (iii) the improved character(s) must be combined with all other desirable characters of the parent; and (iv) the variation must be inherited stably through successive generations by the chosen means of propagation.

Two approaches have been followed to recover somaclonal variation from cultured cells: (1) plants are regenerated from tissue or cells cultured for various periods and screened for the desired traits, and (2) for a

number of characters, such as resistance to a fungal toxin, a herbicide, a pollutant, high salt concentrations and extremes of temperature, the selection might also be made at the cell level. An advantage of the latter method is that it permits screening millions of cells in a very small space with a comparatively small input of effort and resources. However, it cannot be taken for granted that the phenotypes expressed by selected cells would also be expressed by plants regenerated from them. Similarly, certain genes may not be active at the single cell level, and specific tissue functions may occur only when the cell is an integral part of the intact plant. Therefore, the character for which cells have been selected must be rechecked at the whole plant level.

In vitro selection has been mostly made at the callus level. However, selection of single cells is expected to give better results because of a better contact of cells with the selection agent and regeneration of solid mutants.

9.3.1. Selection at plant level

(i) *Sugarcane.* The potential use of somaclonal variation in crop improvement was first demonstrated in sugarcane (Heinz et al., 1977). Plants regenerated from calli of sugarcane exhibit marked phenotypic and genotypic variations, such as cane yield, tillering, fibre content and numerous fine morphological characters. Nickell's group in Hawaii and Krishnamurthi and his co-workers in Fiji isolated, through tissue culture, subclones of established cultivars of sugarcane which showed resistance to eyespot disease (caused by *Helminthosporium sacchari*), Fiji disease (caused by an aphid transmitted virus) and downy mildew (caused by *Sclerospora sacchari*).

Krishnamurthi (1974) and Krishnamurthi and Tlaskal (1974) were able to isolate variants which showed higher resistance to Fiji disease and downy mildew than their parent clones. 'Pindar' is an important sugarcane cultivar of Fiji; it is suited to poor soils but is highly susceptible to Fiji disease. Of the 38 subclones of this cultivar obtained through tissue culture and screened for Fiji disease, four proved resistant. The resistance was maintained through several cane generations in the field. In field trials, three of these lines gave poor yields but one ('Pindar 70-31') performed as well or even better than the original cultivar. Pindar 70-31 also carried high resistance to downy mildew. This work led to the release of a new cultivar 'Ono' with higher yield and greater disease resistance (see Daub, 1986). Heinz et al. (1977) observed that mutagenic treatments did not increase the frequency of variants resistant to eyespot disease over the frequency with which they appeared spontaneously.

Q101 is an agronomically valuable sugarcane cultivar of Australia but for its susceptibility to eyespot disease. Larkin and Scowcroft (1983) isolated somaclones of this cultivar which were highly resistant or nearly immune to the fungal toxin but morphologically identical to the parent variety. Indeed, such small modifications in otherwise outstanding varieties would be especially useful.

(ii) *Potato.* Somaclonal variations have been studied extensively in potato. Most of the popular cultivars of potato are highly heterozygous and, therefore, their improvement by conventional breeding practices is difficult. In this regard tissue culture techniques have proved useful. Shepard et al. (1980) screened about 2500 regenerants from protoplast cultures of the American cultivar 'Russet Burbank' and selected about 60 protoclones which displayed stable improvement of agronomic traits, including resistance to late blight (caused by *Phytophthora infestans*) and early blight (caused by *Alternaria solani*). Similarly, two variants regenerated from a single callus of the cultivar 'Bintje' showed field resistance to late blight (Fourage et al., 1987). Several other potato somaclones expressing higher resistance to common scab than the parental cultivar have also been identified (Thompson et al., 1986a; Fourage et al., 1987). Gunn and Day (1986) isolated protoclones of potato which had tuber yields equal to or better than the parents and were disease resistant.

Thompson et al. (1986a) found potato protoclones which were field-resistant to potato virus Y (PVY) and potato leafroll virus (PLRV). Selection of somaclones allowing low aphid colonization may indirectly contribute to the control of a number of aphid transmitted virus diseases, such as PRLV and PVY. A somaclone variant of the potato cultivar 'Roxane', selected on the basis of its low colonization by *Myzus persicae* (Fig. 9.1), in the green house, showed field resistance to PVY, with a lower percentage of virus-infected tubers than the parental cultivar (see Semal and Lepoivre, 1990).

At the Rothamstead Plant Breeding Station (UK) plants were regenerated from 14 cultivars of potato which varied in such characters as yield, tuber shape and tuber colour (Bright et al., 1985; Wheeler et al., 1985). A white skinned tuber trait, obtained as a variant from the red skinned 'Desiree' variety, has been stably expressed through tuber generations. Chromosomally normal plants with properties differing from the parental line have been obtained and evaluated for field trails. Similarly, a somaclone of the cultivar 'Bintje' showed improved field performance during four successive years, with a yield of marketable tubers (Fig. 9.2) significantly higher (15–20%) than the parent cultivar (Meulemans et al., 1987).

Fig. 9.1. Colonization by *Myzus persicae* of a somaclonal variant (A) compared to the parental potato cv. Roxane (B) (after Semal and Lepoivre, 1990).

Cassells et al. (1983) demonstrated that plants regenerated directly from stem pieces, without a callus phase, also carry considerable variation. Direct regeneration of plants may be a better source of useful variants than protoplast or callus cultures which induce gross abnormalities.

Due to difficulties in performing sexual crosses (limited flowering or sterility of many cultivars) and because of the tetraploid status of most potato genome, no genetic analysis of potato somaclones has been presented so far (Semal and Lepoivre, 1990).

(iii) *Banana*. Tissue culture raised banana plants exhibit a range of morphological variations, including dwarfism, abnormal leaf, pseudostem pigmentation, persistence of flowers and split fingers (Israeli et al., 1991). Drew and Smith (1990) and Smith and Drew (1990a) reported that 22% of the total population of plants regenerated from callus cultures were dwarf, and this trait was retained through five vegetative cycles.

All commercial banana cultivars of Taiwan are susceptible to Race 4 of *Fusarium oxysporum* f. sp. *cubense*. The traditional breeding methods to select *Fusarium* wilt resistant lines of Cavendish banana were unsuccessful (Su et al., 1986). However, several somaclones of this cultivar showed very high resistance to the pathogen (Hwang, 1991). Cultures were initiated from shoot-tips of suckers of the susceptible cultivar and the adventitious shoot buds produced by them were put through 6–7 monthly cycles of multiplication. Of about 20 000 of these plants screened in the field, 6 showed very high resistance to the pathogen (0–10% incidence of disease compared to 60% in the parent cultivar). However, all the 6 variants were agronomically inferior. Through a repeat course of in

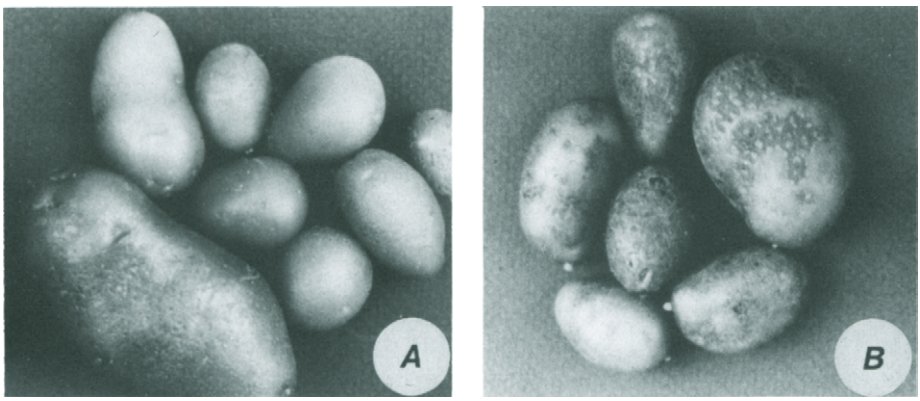

Fig. 9.2. Symptoms of common scab expressed in a somaclonal variant (A) compared to the parental potato cv. Desiree (B) (after Semal and Lepoivre, 1990).

vitro shoot multiplication, as before, from shoot-tips of the resistant variants, Hwang (1991) was able to select at least 3 lines with improved yield. These plants also retained the improved resistance to the disease. One of the three variants (GCTCV-215–1) combined the high resistance trait (5.2–17.2% incidence of disease) with the agronomic traits comparable to that of the parent cultivar.

(iv) *Strawberry.* Toyoda et al. (1991) have reported the selection of strawberry plants with stable resistance to *F. oxysporum* f. sp. *fragriae* by directly transferring the plants regenerated from leaf callus of a susceptible variety to soil infected with the pathogen. In the soil, where almost all the plants of the parent cultivar (control) died after showing the symptoms of the disease, 2 out of the 1225 regenerants grew well and produced 3 daughter generations. Direct application of the pathogen to the roots of the third runner generation plants of the putative somaclone followed by their planting in pathogen infested soil did not induce disease symptoms, and the plants continued to reproduce normally. Under identical conditions all the control plants died within 3 weeks.

(v) *Geranium.* This ornamental plant is traditionally propagated from leaf cuttings. Skirvin and Janick (1976a) compared the plant populations raised from root, stem and petiole cuttings in vivo and those differentiated from their callus cultures. Whereas the plants from in vivo stem cuttings were uniform, those from in vivo root and petiole cuttings and from callus were quite variable. They have released a new scented variety 'Velvet Rose' from a calliclone obtained from the cultivar 'Rober's

Lemon Rose' (Skirvin and Janick, 1976b). The new cultivar has double the chromosome number of the parent and is selected for its general attractiveness and vigour.

(vi) *Paspalum dilatatum*. It is a C_4 forage grass, showing poor seed-set and is susceptible to ergot under New Zealand conditions. Since the productive biotype is an obligate apomict and lacks sexual cross compatibility, the traditional breeding method for its improvement is of little use (Burton, 1962). Attempts to induce variation using irradiation have not been successful (Bashaw and Hoff, 1962). Therefore, its genetic improvement through somaclonal variation would be of particular importance. Davies et al. (1986a) recovered somaclones in batches of plants regenerated from long-term embryogenic callus of this grass. The regenerants showed extensive phenotypic variation in field trials but none displayed resistance to ergot, and seed-set was low. These findings were recently confirmed by Burson and Tischler (1993). While most of the somaclones screened by them displayed low seed germination, one of the variants had significantly higher germination than the common dallisgrass.

(vii) *Tomato.* Somaclonal variants of tomato for several characters, such as fruit colour, plant architecture and characters for mechanical harvesting, have been isolated by Evans and Sharp (1983; see also Evans et al., 1984b; Evans and Bravo, 1986). Detailed genetic analysis of these variants has revealed stable genetic changes caused by single gene mutations, fruit colour being recessive and *Fusarium* resistance as dominant trait. One of the somaclones of tomato, with very high (20%) dry matter content, an enhanced taste and a better texture and colour has been registered as a new variety by DNA Plant Technology Corporation, USA (Evans, 1989).

(viii) *Celery.* Wright and Lacy (1988) observed that the plants regenerated from tissue cultures of celery ranged from highly resistant to highly susceptible to three fungal and one bacterial pathogens. A celery somaclone (UC-T3) exhibiting significantly higher resistance to *F. oxysporum* f.sp. *apii* Race 2 than the parent cultivar (Tall-Utah 527OR) was isolated by Heath-Pagliuso et al. (1988, 1989). Through detailed breeding experiments, Heath-Pagliuso and Rappaport (1990) established that the improved resistance of the UC-T3 was heritable and probably controlled by two genes.

(ix) *Rice.* Several reports of the selection of somaclonal variants from somatic and gametic tissue cultures of rice have been published (Oono,

1978; Chaleff, 1980; Chen et al., 1980; Fukui, 1983). Oono (1988) reported that in 72% of the 762 regenerated lines that were screened, variation were noted for such characters as seed viability, plant height, tillering and chlorophyll deficiency, and somaclonal variants were stable through at least one seed generation. From a single callus formed in seed cultures, Fukui (1983) raised 12 plants with 90% fertility. The progeny of these plants showed variation for leaf colour, early heading, albino and short culm. The author has concluded that the mutations for the four traits occurred independently and successively.

Anther and pollen cultures are a good source of variation (Section 7.9.2). The pollen plants being haploid, stable variants can be obtained in one generation.

Schaeffer et al. (1984) obtained useful variants by selfing anther-derived plants of rice. Chinese workers have produced new varieties of many cereals, including rice, through anther culture. Through a combination of anther culture and somatic tissue culture, Heszky et al. (1992) have evolved an improved variety of rice, 'DAMA'. The 'pollen haploid somaclone' method (PHS method) is summarized in Fig. 9.3.

9.3.2. Selection at cell level

There are two basic strategies for in vitro mutant selection (Gonzales, 1994): (i) Single-step selection: the selection agent is used at a concentration two or three times the MIC (minimum concentration resulting in 100% inhibition). This is the simplest method with clear results and the least likelihood of escapes. (ii) Multiple-step selection: this strategy may be applied where single-step selection does not work. In this approach the concentration of the selective agent is considerably less than the MIC, usually near I_{50} (concentration resulting in 50% inhibition). The concentration is gradually increased in frequent successive subcultures to allow the fast growing resistant cells to outgrow the sensitive wild-type cells. By this process it should be possible to eventually obtain vigorously growing cultures at the inhibitor level well above the MIC established for the original unselected cultures.

(i) *Disease resistance.* Resistance to most plant diseases is not mediated by single genes and may not be detectable in cultures. However, sometimes the toxic effect of a phytotoxin on tissue, cell or protoplast culture is equivalent to its effect on the whole plant (Earle, 1978). Furthermore, if a phytotoxin or toxins is/are the sole determinant of pathogenicity, direct selection of a large population of disease resistant totipotent cells is possible by incorporating the toxin into the medium. This hy-

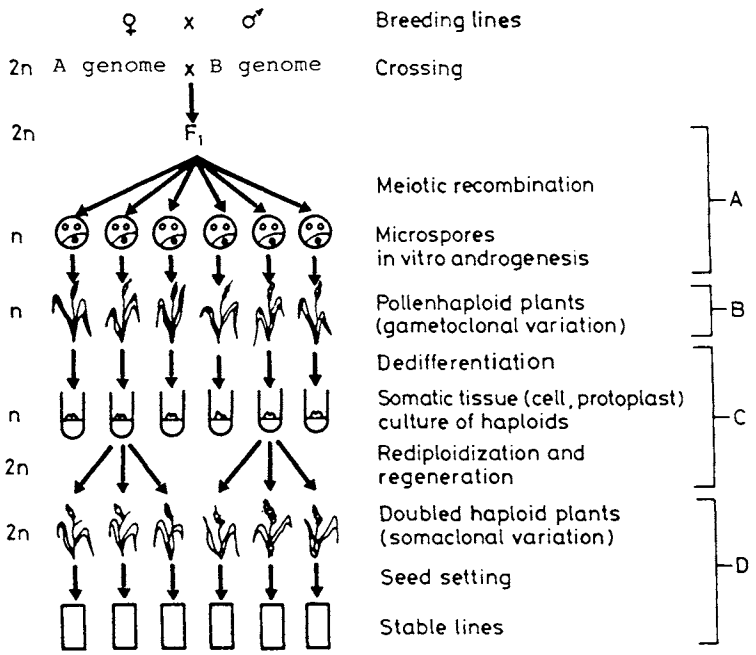

Fig. 9.3. Diagrammatized summary of the pollen haploid somaclonal method (PHS method) for increasing genetic variability in breeding material (after Heszky et al., 1989).

pothesis received experimental support when Carlson (1973) reported that plants regenerated from tobacco protoplasts (treated with the mutagen ethyl methane sulphonate; EMS) selected for resistance to methionine sulfoximine (MSO) showed enhanced resistance to *Pseudomonas tabaci*. While MSO is no longer considered to be an analogue of the *P. tabaci* phytotoxin (Thanutong et al., 1983), this observation raised the possibility of selecting phytotoxin-resistant cells/plants in vitro. Excellent reviews on in vitro selection of disease resistance are written by Daub (1986) and Van den Bulk (1991). Some examples of stable disease resistant somaclones obtained by selection at plant and cell levels are listed in Table 9.3.

Maize plants with Texas male sterile cytoplasm (cms-T) are sensitive to the toxin of *Drechslera maydis* Race T, which causes southern corn leaf blight. With the objective of raising resistant male sterile maize, Gengenbach and his associates made recurrent selection of non-mutagenized embryo callus with progressively higher concentrations of T-toxin and isolated toxin-resistant calli (Gengenbach and Green, 1975) and later whole plants (Gengenbach et al., 1977). These findings were later confirmed by Brettell and Ingram (1979) and Brettell et al. (1980). However,

TABLE 9.3

Some examples of somaclones showing heritable disease resistance obtained by selection at plant level or at cell level

Crop	Pathogen	Selective agent	Culture	Resistance system	References observed
Selection at cell level					
Alfalfa	*Fusarium oxysporum* f.sp. *medicaginis*	Culture filtrate	Callus	Increased resistance	Hartman et al. (1984), McCoy (1988)
Barley	*Helminthosporium sativum*	Crude toxin	Callus	Resistance	Chawla and Wenzel (1987a)
Eggplant	Little leaf disease (mycoplasma-like organism)	Pathogen	Callus from infected tissue	No symptoms	Mitra and Gupta (1989)
Maize	*H. maydis*	Hm toxin	Callus	Resistance	Gengenbach et al. (1977)
Oats	*H. victoriae*	Victorin	Callus	Resistance to victorin	Rines and Luke (1985)
Rape	*Phoma lingam*	Culture filtrate	Suspension cells, embryo cultures	Increased resistance	Sacristan (1982, 1985)
Rice	*H. oryzae*	Crude toxin	Callus	Increased resistance	Ling et al. (1985), Vidyasekaran et al. (1990)
Sugarcane	*H. sacchari*	Toxin	Callus	Increased resistance	Heinz et al. (1977), Larkin and Scowcroft (1983)
Tobacco	*Pseudomonas syringae* pv. *tabaci*	Methionine Sulfoximine	Cells, Protoplasts	No chlorotic haloes	Carlson (1973)
		Crude toxin	Callus	Resistance	Thanutong et al. (1983)
Tobacco	Tobacco mosaic virus	Virus	Callus from infected tissue	Reduced virus multiplication and restricted translocation	Murakishi and Carlson (1982), Toyoda et al. (1985, 1989)

TABLE 9.3 (*continued*)

Crop	Pathogen	Selective agent	Culture	Resistance system	References observed
Tomato	*F. oxysporum* f.sp. *lycopersici*	Culture filtrate	Callus culture	Tolerance to filtrate	Scala et al. (1984)
		Fusaric acid	Protoplasts	Resistance	Shahin and Spivey (1986)
Tomato	Tobacco mosaic virus	Virus	Infected explants	Tolerance	Cassells et al. (1986)
Wheat	*H. sativum*	Crude toxin	Callus	Resistance	Chawla and Wenzel (1987b) Chawla and Kole (1990)
Selection at plant level					
Banana[a]	*F. oxysporum* f.sp. *cubense*	–	Meristem	Increased resistance	Hwang and Ko (1988), Hwang (1991)
Celery	*F. oxysporum* f.sp. *apii*	–	Embryogenic suspension culture, callus	Increased resistance	Ireland and Lacy (1987), Wright and Lacy (1988), Heath-Pagliuso et al. (1988), Heath-Pagliuso and Rappaport (1990)
Maize	*H. maydis*	–	Callus	Resistance to Hm toxin	Brettell et al. (1980)
Peach[a]	*Xanthomonas campestris* pv. *pruni*	–	Callus	Increased resistance	Hammerschlag and Ognjanov (1990)
Potato[a]	*Alternaria solani*	–	Protoplasts	Smaller lesions	Matern et al. (1978)
Potato[a]	*Phytophthora infestans*	–	Protoplasts	Increased resistance	Shepard et al. (1980)
			Protoplasts, callus, explants	Increased resistance	Meulemans and Fourage (1986), Meulemans et al. (1986)
Potato[a]	Potato virus X, Potato virus Y	–	Callus, explants	Field resistance	Cassells et al. (1986, 1987)

Potato[a]	*Streptomyces scabies*	–	Explants	Increased resistance	Evans et al. (1986)
Rape*	*Phoma lingam*	–	Callus, embryogenic suspension	Increased tolerance	Sacristan (1982, 1985)
Rice*	*H. oryzae*	–	Callus	Resistance	Ling et al. (1985)
Strawberry[a]	*F. oxysporum* f.sp. *fragariae*	–	Callus	Resistance	Toyoda et al. (1991)
Sugarcane[a]	*Fiji virus*	–	Callus	Increased resistance	Krishnamurthi and Tlaskal (1974), Heinz et al. (1977)
Sugarcane*	*H. sacchari*	–	Suspension culture, Explants	Increased resistance	Larkin and Scowcroft (1983)
Tomato	*Tobacco mosaic virus*	–	Explants	Resistance	Barden et al. (1986)
Tomato	*F. oxysporum* f.sp. *lycopersici* race 2	–	Callus	Resistance	Smith and Murakishi (1987), Miller et al. (1985), Evans (1987)
Tomato*	*F. oxysporum* f.sp. *lycopersici* race 2	–	Protoplasts	Resistance	Shahin and Spivey (1986)

[a]Transmitted after vegetative propagation.
*In the studies marked with an asterisk, disease resistant plants were obtained from non-selected control plants of in vitro selection experiments.

in both cases toxin-resistance was accompanied by restoration of male fertility. In a later experiment, Umbeck and Gengenbach (1983) found that of the 169 plants regenerated from calli of T-toxin sensitive cms maize plants 2 were unexpectedly male fertile and toxin-sensitive. However, R_1 progeny of these plants reverted to male fertile-toxin resistant and male sterile-toxin sensitive type plants, suggesting that the two traits are closely linked. Restriction endonuclease pattern of mitochondrial DNA revealed significant changes in the mitochondrial DNA of the regenerated variant plants (Gengenbach and Connelly, 1981; Kemble et al., 1982; Kemble and Pring, 1982).

Fusarium wilt resistant somaclones of tomato could be isolated after challenging the cotyledon protoplasts with fusaric acid, a non-specific toxin, as well as from non-selected calli of a susceptible variety (Shahin and Spivey, 1986). The resistance, conferred by a single dominant gene, was transmitted through several sexual generations.

Although it is very attractive to enrich the population of cells with desirable genotypes through in vitro selection, often the somaclones from cells selected for resistance to toxin or fungus culture filtrate do not exhibit stable resistance to the pathogen (Koike et al., 1991; Toyoda et al., 1989). For example, celery cells resistant to fusaric acid did not regenerate plants with higher resistance to the *F. oxysporum* f. sp. *apii* race 2 (FOA_2) than the control, suggesting that the principal mode of infection by FOA_2 does not involve a host specific toxin (Heath-Pagliuso et al., 1988). In contrast, selection of regenerated plants in FOA_2-infested soil yielded two plants showing significantly higher resistance to the fungus than the parent cultivar. The new variation was heritable, controlled by two dominant genes (Heath-Pagliuso and Rappaport, 1990).

(ii) *Herbicide resistance.* Herbicide resistance is important not only for plant breeding purposes but also as a selective marker in genetic engineering. Herbicide resistant plants of some species have been produced by genetic engineering (Chapter 14). Somaclonal variation is a practical alternative approach for selection of herbicide resistant mutants in cases where transgenic plants are difficult to produce.

Selection of herbicide resistant phenotypes in the field is complicated by difficulties in applying high and uniform selection pressure. Comparisons of herbicide action at cell, tissue and plant levels have indicated the possibility of selecting for herbicide resistance in tissue cultures.

The feasibility of developing herbicide-tolerant plant varieties through in vitro selection was first demonstrated by Chaleff and Parsons (1978). They selected picloram-resistant tobacco cell lines by exposing cell suspension to 500 μM of the herbicide. In some of the plants regenerated

from selected cell lines herbicide tolerance was inherited as dominant or semi-dominant alleles of single nuclear genes.

Chlorsulfuron (CS) and sulfometuron methyl (SM), sulfonylurea type of herbicides, inhibit the biosynthesis of acetolactate synthase (ALS). Tobacco plants resistant to CS and SM were isolated by callus selection (Chaleff and Ray, 1984). One of the somaclones resistant to CS was shown to possess an altered form of ALS which was far less sensitive to inhibition by the two sulfonylurea herbicides than that from normal cells (Chaleff and Mauvais, 1984). The CS resistance was inherited as a single dominant or semi-dominant trait. Plants homozygous for CS resistance showed 100-fold more resistance than normal plants. Recently, barley variants resistant to CS under greenhouse conditions have been isolated (Baillie et al., 1993). *Lotus corniculatus* lines showing genetically stable resistance to the sulfonylurea herbicide, harmony, have been isolated through sequential selection at the callus, shoot and whole plant levels (Pofelis et al., 1992).

Bensulfuron methyl (BSM), another member of the sulfonylurea family of herbicides, is highly toxic to annual and perennial dicotyledonous weeds. Its toxicity to rice is not severe but it effects the initial growth of rice plants by inhibiting root elongation. Terakawa and Wakasa (1992) selected BSM-resistant calli of rice by exposing the seed-derived callus to 0.1 mM BSM. Of the 19 plants regenerated from 6 resistant calli, 2 showed inheritance of the BSM-resistance trait up to the R_2 generation. The resistance was controlled by a dominant nuclear gene.

(iii) *Salt tolerance.* Salt tolerance in crop plants is an increasingly desirable characteristic not only because of limited water supply in the world but also because of salinization of irrigated soils. Since traditional breeding methods have been slow to yield substantial improvement in salt tolerance, the alternative approach of utilizing plant cell culture and regeneration of plants from the selected cell lines has received increased attention.

Initial efforts to produce salt tolerant plants through in vitro selection was not successful because of the inability to regenerate vigorous and fertile plants from selected cell lines (Raghava Ram and Nabors, 1985; Winicov, 1994). Although we still do not understand the mechanism by which plants can acquire incremental improvement in their salt tolerance, recently it has been possible to regenerate fertile plants from cells after selection on culture media containing additional salt and to demonstrate that the salt tolerant trait can be sexually transmitted. Thus, the generation of salt tolerant plants through tissue culture appears to be a viable alternative to classical breeding.

Nabors et al. (1975, 1980) isolated tobacco cell lines tolerant to high NaCl levels (0.88%). Regenerated plants retained tolerance through two successive sexual generations.

Winicov (1991) regenerated salt tolerant alfalfa plants which were morphologically indistinguishable from their salt sensitive parents, and the growth characteristics of the F_2 seedlings were comparable to the parent seedling. Winicov (1994) has recommended that to avoid the appearance of undesirable variants along with the desired salt tolerance the alfalfa callus should be exposed to MIC_{100} salt concentration within 3 months of initiation. The selected salt tolerant lines are expected to grow better on salt containing callus proliferation medium than on salt-free callus proliferation medium. Cell lines that are stably salt tolerant will not lose their ability to grow at a high salt level even after 2 months growth on control medium. Increased vigour has been observed in several plants selected through survival on salt medium in vitro followed by immediate regeneration (McHughen, 1987). High salt medium has helped achieve plant regeneration in long-term cultures of rice and to restore the lost regeneration potential of old cultures (Binh and Heszky, 1990). Interestingly, all the plants regenerated on this medium were salt tolerant.

9.4. ORIGIN OF SOMACLONAL VARIATION

The variations observed in the plants regenerated from cultured cells are derived from two sources (Fig. 9.4): (i) some of the variations could be revelation of the inherent cellular heterogeneity of the explant, and (ii) culture conditions may bring about new genetic changes.

9.4.1. Pre-existing variability

Normal plant development (in about 90% angiosperms) is accompanied by a range of direct changes in nuclear DNA (D'Amato, 1990). Consequently, in the majority of plants, mature and differentiated tissues, such as cortex and pith, exhibit considerable variations in the chromosomal constitution of their cells (Bennici and D'Amato, 1978). In apical meristems (root tips and shoot tips), where DNA synthesis is immediately followed by karyokinesis and cytokinesis (normal cell cycle), cells are maintained at a uniform diploid level (Fig. 9.5). However, the derivatives of these meristematic cells, during their subsequent differentiation do not divide by normal mitosis but may undergo DNA duplication and endoreduplication (see Figs. 9.5, 9.6). The varying degrees of endoreduplication results in somatic cells with 4C, 8C or even higher levels of DNA (see Fig.

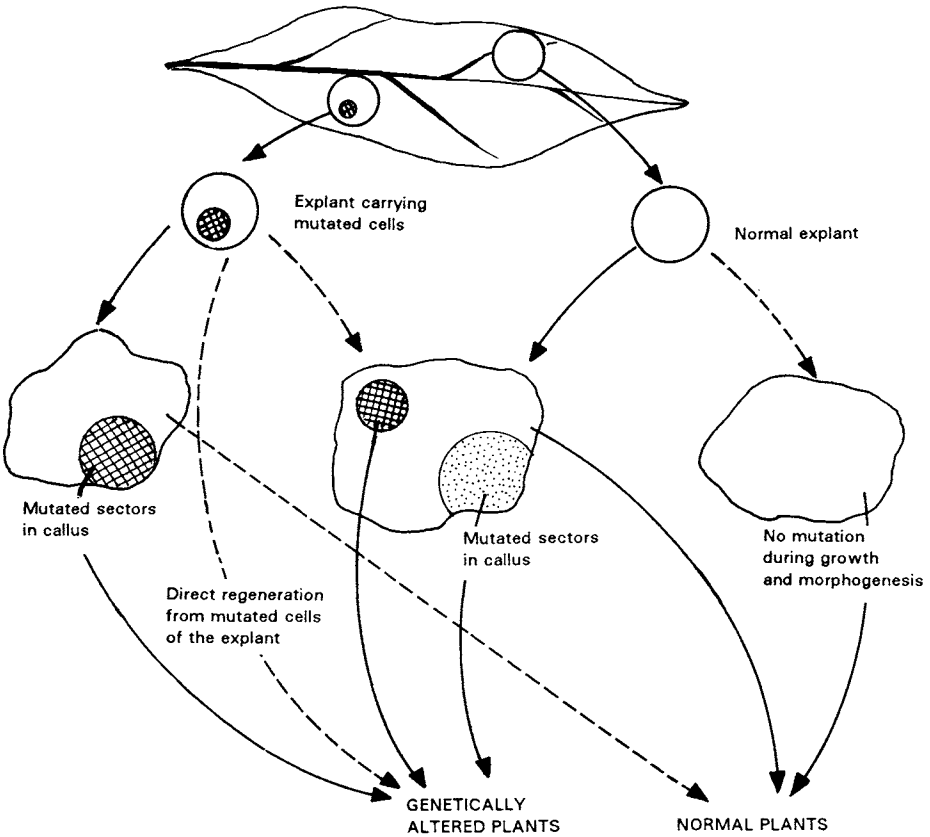

Fig. 9.4. Origin of genetic variation in plants regenerated from explant or callus cultures.

9.5). This phenomenon of polyplodization of body cells is termed polysomaty.

The widespread occurrence of polyploidy in differentiated cells may suggest that endopolyploidy of somatic cells is a general prerequisite for histological differentiation. However, this is not true because in some non-polysomatic species, such as *Helianthus tuberosus* (Partanen, 1959), *H. annuus* (Kupila, 1958; Butcher et al., 1975), *Crepis capillaris* (Fenzl and Tschermak-Woess, 1954), *Dendrophthoe falcata* (Johri and Nag, 1974) and *Lilium longiflorum* (Sheridan, 1975), normal tissue differentiation occurs in the complete absence of endopolyploidy. Polyploidy may, therefore, be an outcome of the process of differentiation. One possible significance of polysomaty could be the blockage of further division of the differentiated cells under normal conditions.

Under a normal situation, the genetic variability induced in somatic cells by polysomaty or any other kind of genetic changes remains unno-

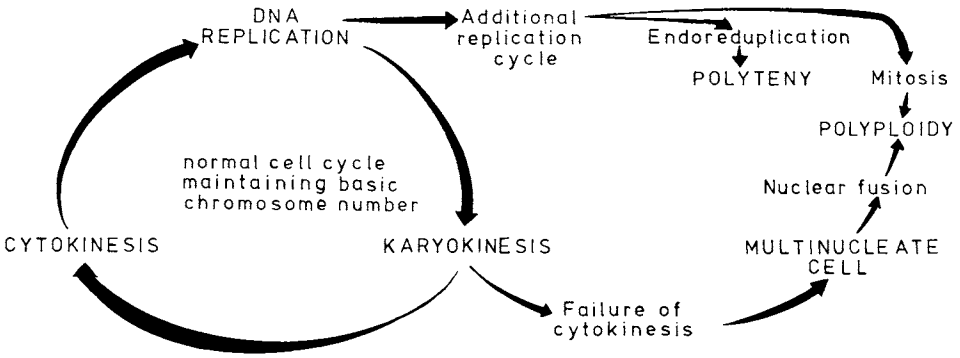

Fig. 9.5. Normal cell cycle and deviations from it, leading to polyploidization of cells in nature (after Partanen, 1965).

ticed as these cells do not divide. However, under culture conditions these cells may be induced to divide and undergo re-differentiation and express the inherent variability at whole plant level.

Torrey (1965) presented autoradiographic evidence to suggest that the first set of tetraploid cells observed in root cultures of pea, a polysomatic species, are those already present in the explant. In the first mitoses in cultures, diploid cells divided with 2n chromosomes (2 chromatid chromosomes) whereas reduplicated cells divided with 2n diplochromosomes (4 chromatid chromosomes), 2n quadruplochromsomes (8 chromatid chromosomes) or 2n polychromosomes (more than 8 chromatids, polyteny). Diplo- and quadruplochromosome mitoses have been commonly observed in the first phase of in vitro growth of tissues, such as stem pith of *Nicotiana* (Naylor et al., 1954; Patau and Das, 1961), stem internodes of haploid *Pelargonium* (Bennici et al., 1968) and the seedling callus of *Haplopappus gracilis* (Bennici et al., 1971).

Since some parts of the plants are more liable to undergo genetic changes in nature than the others, the extent of variation contributed by the donor plant is expected to vary with the explant. Accordingly, meristematic or embryonic tissues are likely to yield most stable calli and regenerated plants. The occurrence of somaclonal variation in the cultures of apical and lateral meristems of *Ipomoea batatas* is a rare exception (Moyer and Collins, 1983). All the plants of pineapple obtained from syncarp (compound ovary) or slip (small shoot which appears just below the fruit) callus were variants while only 7% of the plants obtained from crown (shoot at the top of the fruit) callus were variable (Wakasa, 1979). Similarly, many of the plants regenerated from seed callus of *Cymbopogon* sp. were atypical but those obtained from inflorescence callus closely resembled the parents, with only a negligible variation (Jagdish Chandra

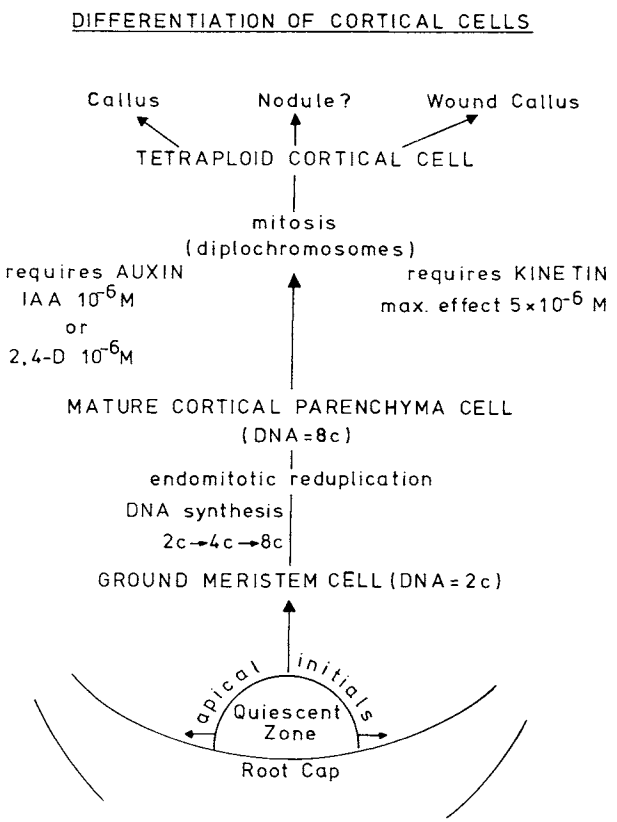

Fig. 9.6. Diagrammatic representation of a longitudinal section of a root to trace the derivation of polyploid-cortical cells from diploid cells in the root apex through endomitosis. Auxin and cytokinin induce divisions in these cells giving rise to a tetraploid cell population (after Torrey, 1965).

and Sreenath, 1982). The plants obtained indirectly from spadix explants of *Anthurium scherzerianum* were less variant than those produced from leaf segments (Geier, 1987). In potato, cotyledon-derived protoplasts produced more tetraploids than the leaf-derived protoplasts (Osifo et al., 1989).

Another type of pre-existing chromosomal variability, viz. aneusomaty, occurs only rarely in plants of hybrid origin (*Saccharum officinarum*; Heinz et al., 1969) and polyploids of recent origin (D'Amato, 1985). Every individual of sugarcane clone H50-7209 shows chromosome number mosaicism ($2n$ = 108–128; see D'Amato, 1977). In these plants the apical meristems and, consequently, the mature tissues comprise a mosaic of cells with varying proportion of different aneuploid chromosome numbers. The aneusomatic condition is transmitted to, and generally en-

hanced in, callus cultures derived from such tissues (Heinz et al., 1969; Cavallini and Lupi, 1987). Breakdown of genetic chimeras during callus or direct differentiation of adventive organs may be another source of somaclonal variation, particularly in vegetatively propagated species (Preil, 1986).

The extent of variation originating from the explant is dependent on, among other things, the age (Brossard, 1975; Castorena Sanchez et al., 1988) and type of tissue and organs (Sree Ramulu and Dijkhuis, 1986), genotype of the donor plant (Krikorian et al., 1993) and even the mode of cultivation of the donor plant (Pijnacker et al., 1989).

9.4.2. In vitro induced variability

The plant genome is under continuous flux which helps the plant adjust to changes in environmental conditions (Walbot and Cullis, 1985; Cullis, 1981). The tissue culture system is a stressful environment for the plant cells (De Klerk, 1990; Phillips et al., 1994) that could result in adaptive changes of genetic or epigenetic nature. Direct evidence for tissue culture-induced variation is the occurrence of chromosomal changes in the cultured tissues of non-polysomatic species, such as *Crepis capillaris* (Sacristan, 1971; Hahne and Hoffmann, 1986), strains of sunflower (Butcher et al., 1975), and in the cultures derived from single cells (Cooper et al., 1964; Mahfouz et al., 1983) or protoplasts (Karp et al., 1982). Generally, less variations are found in the plants than in the callus from which they are derived because in mixed population of cells, with different ploidies, euploid cells tend to be more regenerative than aneuploid cells. Several factors have been shown to influence the induction of variability in tissue cultures.

(i) *Culture medium.* It has been assumed that certain constituents of the culture medium, particularly certain growth regulators are mutagenic (Vajrabhaya, 1977; George and Sherrington, 1984). Torrey (1965) observed that in the cultures of pea root segments on a medium with 2,4-D as the sole hormone only diploid cells divided but when the medium contained kinetin and yeast extract, in addition to 2,4-D, the tetraploid cells were selectively induced to divide.

Direct as well as inverse correlations have been reported between polyploidy in tissue cultures and the presence of 2,4-D in the medium. Whereas some authors (Mitra and Steward, 1961; Melchers, 1965; Sunderland, 1977) regard this auxin as a direct inducer of polyploidy, others consider it as a factor selectively favouring the division of polyploid cells. Sunderland (1977) observed that in a medium containing 2,4-D

the suspension cultures of *Haplopappus* changed from a wholly diploid state to a wholly tetraploid state within a period of 6 months. This change was much slower if the medium contained NAA in place of 2,4-D. Similarly, chromosomal abnormalities in *Nigella sativa* tissues occurred at maximum frequency when 2,4-D, rather than IAA or NAA, was present in the medium together with kinetin (Chand and Roy, 1980). Increase in the number of sister chromatid exchanges (SCEs) per chromosome was noted in garlic roots treated with 5–15 μM 2,4-D (Dolezel et al., 1987). In wheat, 2,4-D did not induce such changes but 2,4,5-T at concentrations higher than 2 mg l^{-1} caused dramatic increases in SCEs which was partially suppressed by simultaneous addition of kinetin (Murata, 1989).

Kallak and Yarvekylg (1971) noted an inverse relationship between the concentration of 2,4-D and the degree of polyploidization of cultured cells of pea. According to these authors, 2,4-D at an auxetic (hormonal) concentration (0.25 mg l^{-1}) increased polyploid mitoses and decreased diploid mitoses but when applied at a herbicidal concentration (20 mg l^{-1}) it favoured division in diploid cells. Interestingly, the mitotic index in the callus was comparable at both the concentrations. An increase in the population of diploid cells, by selective elimination of higher ploidy cells, with an increasing concentration of 2,4-D was also observed in suspension cultures of *Haplopappus gracilus* and *Vicia hajastana* (Singh and Harvey, 1975b). In some cases cytokinin increased the extent of polyploidy in callus cultures (Torrey, 1958; Ghosh and Gadgil, 1979; Wright and Northcote, 1973) but in others either it had no effect (Butcher et al., 1975) or even reduced polyploidy and other karyotypic variability (Bennici et al., 1971; Vanzulli et al., 1980; Dolezel and Novak, 1985; Ashmore and Shapcott, 1989). Some of the potato plants (4.3%) regenerated from internode calli on medium containing zeatin showed increased resistance to *Phytophthora infestans* but none of the plants regenerated in the presence of 2ip showed increased resistance (Meulemans et al., 1986).

Besides the growth substance, some basic constituents of culture media also influence the cytological behaviour of cultured cells. In a mixed culture of a predominantly diploid and a hypertetraploid carrot cell lines the selective advantage of the latter line to proliferate on standard medium was substantially reduced in nitrogen and phosphate limiting medium (Bayliss, 1977, 1980). In suspension cultures of *Datura innoxia* organic nitrogen favoured the proliferation of diploid and tetraploid cells whereas inorganic nitrogen favoured the multiplication of haploid cells (Furner et al., 1978). Similarly, whereas B_5 medium selected for diploids, MS medium selected for tetraploid cells in *Haplopappus gracilis* (Singh,

1975b). Whites medium, which is a low salt medium, prevented the loss of embryogenic potential of carrot cells that occurred on MS medium (Smith and Street, 1974). An overdose of $CaCl_2$ in the medium increased the frequency of gross aberrations among regenerants of the potato cv 'Bintje' (Semal and Lepoivre, 1990).

(ii) *Growth pattern and mode of regeneration*. The nature of the callus may affect the variability observed in the regenerated plants. A true callus is a mass of dedifferentiated cells proliferating in an unorganized manner, and is likely to introduce considerable variability. However, in several monocots the callus often represents a mass of suppressed organs or proembryos rather than completely unorganized tissue (Humault, 1979; Wernicke et al., 1982; Wernicke and Gorst, 1987). Such calli are likely to retain a high degree of karyotypic constancy. This may explain the relative genetic stability of tissue cultures and regenerants reported in anthuriums (Geier, 1987), asparagus (Becker and Reuther, 1986), barley (Karp et al., 1987), daylily (Krikorian et al., 1981), guinea grass (Hanna et al., 1984), lily (Sheridan, 1975), maize (Edallo et al., 1981; McCoy and Phillips, 1982) and wheat (Chu et al., 1987). The same explanation is probably true for the stability observed in the cultures derived from zygotic embryos of conifers, which represent masses of proliferating embryos rather than disorganized calli (Papes et al., 1983; Schuller et al., 1989). In *Anthurium scherzerianum*, selective transfer, on to the same medium, of callus portion with or without shoot initials led to karyotypically stable and highly morphogenic sublines and highly variable, non-morphogenic sublines, respectively (Geier, 1988).

It has been suggested that regeneration via embryogenesis has better chances of obtaining genetically uniform plants than through organogenic differentiation (Vasil, 1987). However, this is not always true. Plants raised through somatic embryogenesis in petiole cultures of fennel exhibited considerable variability in various morphological parameters (Humault and Desmaret, 1990). High chromosome variability was also observed in embryogenic cultures of celery (Orton, 1985, 1987) and maize (Armstrong and Phillips, 1988). All the somatic embryos of *Bellevalia romana* were uniformly diploid up to the globular stage but during their further development chromosome mosaicism occurred (Cavallini et al., 1987).

Growth of unorganized callus was thought to be necessary for induction of variability but recent studies suggest that genetic variability is even present in population of plants directly regenerated from explants adventitiously (Cassells et al., 1983; Evans and Bravo, 1986; Bhojwani and Arora, 1992).

(iii) *Length of culture period and frequency of subculture.* Variant karyotypes commonly accumulate with increasing age of callus and, consequently, the proportion of variant plants produced during successive passages generally also increases (Geier, 1991). An increase in the frequency of regenerants displaying aneuploidy and/or chromosome structural changes with the length of culture period has been observed in garlic (Novak, 1980), maize (Lee and Phillips, 1987; Benzion and Phillips, 1988), oat (McCoy et al., 1982) and triploid ryegrass (Ahloowalia, 1983). In most of these cases the proportion of karyotypically normal plants was nearly 100% during the first passage.

There is evidence to suggest that frequent transfers compared to extended subculture intervals, yield more stable cultures. A higher degree of diploidy could be maintained in suspension cultures of alfalfa (Binarova and Dolezel, 1988), carrot (Bayliss, 1980; Bayliss and Gould, 1974), *Nigella sativa* (Ghosh and Gadgil, 1980) and tobacco (Evans and Gamborg, 1982) through frequent transfers . In *Begonia rex*, callus ageing resulted in increased aneuploidy and polyploidy (Cassells and Morrish, 1987). In suspension cultures of *Haplopappus* maintained by regular subcultures every 2 days, Singh and Harvey (1975a) observed equilibrium in the cell population of 93% diploid and 7% tetraploid cells over 300 days. Longer passages enhanced the range of nuclear abnormalities.

(iv) *Ploidy and genotype.* Genotype of the parent plant is a strong determinant of variability in cultures. Skirvin and Janick (1976a) noted that the frequency of variation observed in the regenerants of *Pelargonium* was dependent on the variety. Cummings et al. (1976) found differences in the extent of variation induced in different varieties of flax.

Similarly, ploidy of the donor plant also determines the susceptibility of cells to in vitro changes. Generally, ploidy levels lower than the usual ploidy level of the respective species prove to be more or less unstable. Sacristan (1971) compared the cytological changes in long-term cultures of haploid and diploid strains of *Crepis capillaris* and noted that diploidization in haploid tissues was more common than the occurrence of tetraploids in diploid lines.

Where comparison has been made between polyploid and diploid genotypes of the same species generally the former tended to show higher variation in culture. For example, 12 out of 47 regenerants from two 4X ryegrass genotypes lost up to three chromosomes while all the regenerants of the 2X genotypes retained the diploid chromosome number (Jackson and Dale, 1988). Similarly, cultures of a diploid line of potato did not give rise to any morphological variation whatsoever (Wenzel et al., 1979), whereas somaclones of the tetraploid variety 'Russet Burbank'

varied widely with respect to a large number of traits, many of them being of agronomic importance (Thomas et al., 1982). In contrast, autotetraploids of *Phlox drummondi* were found to be karyologically more stable than the diploids (Raja et al., 1992).

(v) *Physical factors.* Besides its chemical composition, the physical state of the medium also influences the cytological behaviour of the cultured cells. In *Hevea*, polyploidy increased when cells were cultured in suspensions but decreased when they were recultured as callus on a solid medium (Wilson et al., 1976).

Culture temperature can influence the rate of mutation. Tobacco callus incubated at 35°C remained predominantly diploid, while the same tissue cultured at 25°C showed marked karyological instability and became mainly tetraploid (Binns and Meins, 1980). In *Lilium longiflorum* the occurrence of albino seedlings from somatic embryos increased as the incubation temperature was raised above 10–15°C (Jackson and Dale, 1988).

9.5. MECHANISMS UNDERLYING GENETIC VARIATION

9.5.1. Changes in ploidy level

Ploidy changes is the most frequently observed chromosomal abnormality in cultured cells and the plants regenerated from them (D'Amato, 1985; Geier, 1991). With respect to the number of chromosomes two main types of abnormalities occur in cultured cells: (a) euploidy: increase in chromosome number in simple multiples of the basic chromosome number ($2n$, $3n$, $4n$, $5n$, etc.), and (2) aneuploidy: cells with chromosome numbers which are not simple multiples of the basic chromosome number. Geier (1991) scored 306 papers describing chromosomal variability in tissue cultures, of which 295 papers reported numerical changes (euploidy 239; aneuploidy 205).

Formation of a restitution nucleus due to the failure of spindle formation and chromosome lagging at anaphase (Bayliss, 1973; Knosche and Gunther, 1980; Dobel, 1983; Dolezel and Novak, 1985; Zhang et al., 1987) and the fusion of spindles during synchronous divisions in multinucleate cells (Mitra et al., 1960; Mitra and Steward, 1961) are common sources of the occurrence of euploid cells of the 4,8,16... series in tissue cultures. Fusion of nuclei may even occur during interphase (Constabel et al., 1975). Euploid cells of odd series ($3n$, $5n$, $7n$, etc.), which are also commonly encountered in tissue cultures (Mitra and Steward, 1961; Fox, 1963; Shamina, 1966; Sacristan and Melchers, 1969) may arise through

nuclear fusion (Sunderland, 1977) or genome segregation during polyploid mitosis (D'Amato, 1977).

Haploid cells, sometimes found in calli of diploid origin (Mitra and Steward, 1961; Gupta, 1971; Singh et al., 1972; Bennici et al. 1976, Roy, 1980; Singh, 1986; Zhang et al., 1987), probably arise through somatic pairing and reduction, a process that is well documented in intact root meristems (Mehra, 1986), and has been shown to occur at least in some callus cultures (Mitra and Steward, 1961). Nuti Ronchi et al. (cited in Nuti Ronchi, 1990) have observed all stages of meiosis in cell cultures of carrot.

With the exception of plants of hybrid origin (*Saccharum officinarum*; Heinz et al., 1969) and polyploids of recent origin, aneuploidy normally does not occur in nature. However, aneuploidy in cultured cells is not a rare feature. In the pith tissue cultures of *Nicotiana glauca*, on a 2,4-D containing medium, extensive nuclear fragmentation (amitosis) was noted during the first 2–6 days of culture (Nuti Ronchi et al., 1973). This resulted in multinucleate cells having nuclei of varying sizes. Normal mitoses in such cells is expected to produce cells with a wide range of chromosome numbers. This has been actually observed in suspensor cultures of *Phaseolus coccineus* (Bennici et al., 1976) (Fig. 9.7) and cotyledon cultures of *Vicia faba* (Cionini et al., 1978), as early as the first wave of regular mitosis in the explant. However, many of the aneuploid cells may be eliminated at later stages through mitotic selection in favour of diploid cells (Cionini et al., 1978).

The selection in favour of certain type of cells in cultures plays a significant role in establishing dominant karyotype or modal chromosome number. Singh (1975b) isolated 4-, 6-, 16-chromosome lines from tissue cultures of *Haplopappus gracilis*. In prolonged cultures, on B_5 medium supplemented with 2,4-D, the 4- and 6-chromosome lines were stable whereas the 16-chromosome line gradually lost chromosomes, and a stable karyotype with 13 chromosomes evolved. In mixed cultures of diploid and tetraploid cell lines of carrot (both lines show identical growth rates in monocultures) the frequency of tetraploid cells gradually increased and, finally, the cultures attained a tetraploid mode (Smith and Street, 1974).

The regeneration process itself acts as a screen to eliminate a portion of varying karyotypes. Generally, a strong selection acts in favour of diploids (Hahne and Hoffmann, 1986; Sengupta et al., 1988; Geier et al., 1992) or at least euploids (Taniguchi and Tanaka, 1989) during plant regeneration from callus and suspension cultures. Selection may occur at different stages in the regeneration process. Some of the cells with chromosomal variation may be totally impaired in their regeneration process

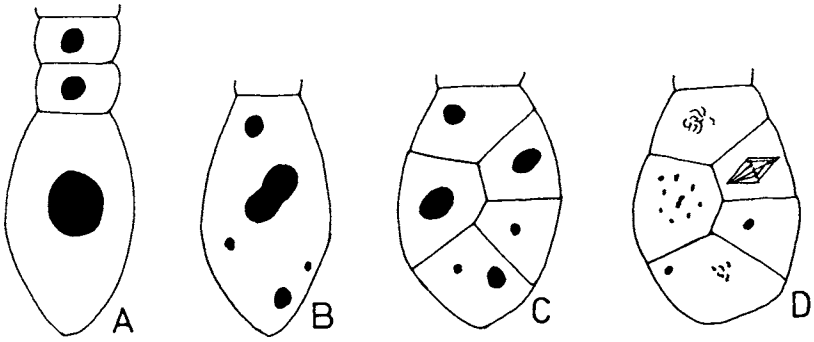

Fig. 9.7. Diagrammatic representation of nuclear irregularities reported to occur in suspensor cells of *Phaseolus coccineus*. (A) Part of the suspensor at the time of culture. (B) Fragmentation of the nucleus in the basal cell. (C) Cellularization of the basal cell; the resulting cells include nuclei of different sizes. (D) Some of the aneuploid cells are dividing (after Bennici et al., 1976).

(Murashige and Nakano, 1965; Torrey, 1967). The plants regenerated from embryogenic cell suspensions of *Euphorbia pulcherrima*, which showed DNA content ranging from 2C to 32C, were highly uniform with regard to their phenotype and ploidy level (Geier et al., 1992). The shoots regenerated from potato protoplasts showed a significant difference in their rootability depending on whether they were euploid (well rooting) or aneuploid (poorly rooting) (Fish and Karp, 1986).

In some cases, where explants or calli are aneusomatic and the regenerants are of multicellular origin, the resulting plants may be a mosaic of cells with different euploid and aneuploid chromosome number (Bennici, 1979; Lupi et al., 1981; Natali and Cavallini, 1987). The wheat plants regenerated from mesocotyl segments of Durum wheat, within 10–15 days of culture, showed chromosome number mosaicism in root-tips and shoot-tips. The constituent cells of the mosaic were hypohaploid, haploid and hypodiploid besides being diploid (Bennici and D'Amato, 1978). The aneusomaty persisted until an advanced stage of spike development but was completely eliminated before meiosis in micro- and megaspore mother cells (Lupi et al., 1981).

9.5.2. Changes in chromosome structure

The stability of chromosome number in cultured cells does not rule out their karyotypic instability. Structural rearrangements involving gain or loss of chromosome segments can lead to entirely altered karyotype while maintaining the original number of chromosomes (Lee and Phillips, 1988). *Crepis capillaris* has three pairs of chromosomes which can be

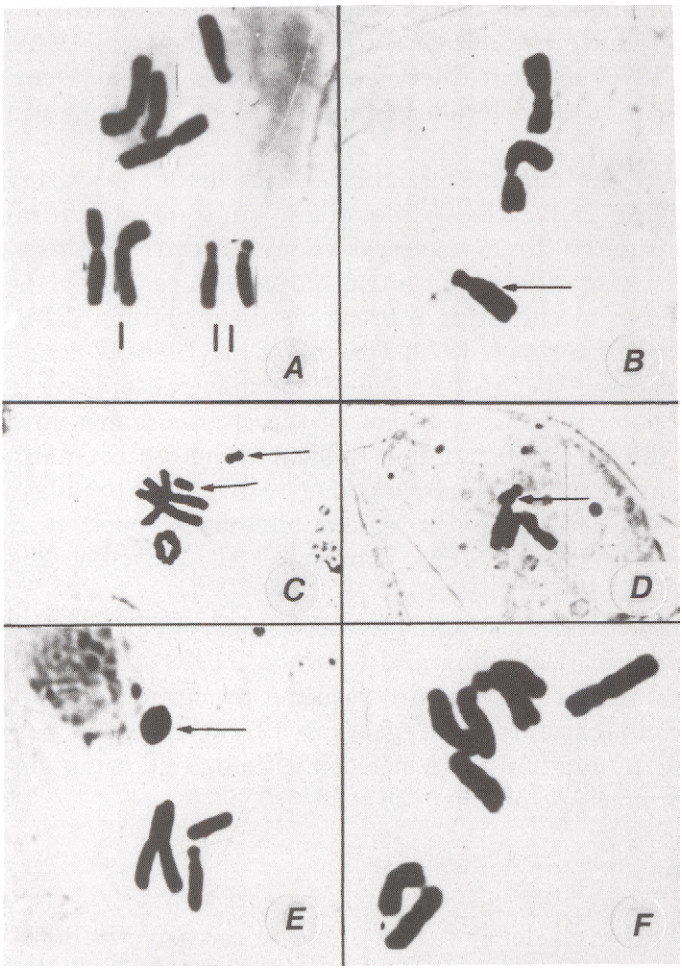

Fig. 9.8. Metaphase figures from cells of *Haplopappus gracilis* grown in suspension cultures. (A) A karyotype of a diploid cell ($2n = 4$); chromosome II has a satellite. (B) A monosomic cell for chromosome II. (C) A cell showing chromosome fragments (arrow marked). (D) A diploid cell with a deleted chromosome II (arrow marked). (E) An aneuploid cell with a ring chromosome. (F) A tetraploid cell (after Singh and Harvey, 1975a; courtesy of Dr B.D. Singh, India).

easily distinguished on the basis of their morphology. Pair 1 comprises long chromosomes (L), pair 2 has medium size satellite chromosomes (Sat) and pair 3 consists of small chromosomes (S). In long-term cultures the frequency of rearrangement varied with the chromosome: 47% for L chromosomes, 82.3% for Sat chromosomes and 64.6% for S chromosomes (Sacristan, 1971). In tissue cultures of *Haplopappus gracilis*, the occurrence of acentric fragments, microchromosomes, deleted chromosomes, dicentric chromosomes and ring chromosomes. (Fig. 9.8) was very fre-

quent (Singh, 1975a,b; Singh and Harvey, 1975a). Reciprocal and non-reciprocal translocations have been observed in potato (Shepard, 1982), ryegrass (Ahloowalia, 1976) and oat (McCoy et al., 1982). Wheat tissue cultures showed duplication or deletion of large sections of chromatin (Davies et al., 1986b).

Structural changes in chromosomes originate from breakage during the various stages of the cell cycle. While many kinds of structural changes are stably propagated through successive mitotic cycles, translocations leading to the formation of dicentric chromosomes can bring about continuing variation by initiating a breakage fusion bridge (BFB) cycle (Kao et al., 1970b; Sunderland, 1973; Toncelli et al., 1985). Lee and Phillips (1988) have proposed that culture conditions may cause delay in DNA synthesis in the heterochromatin region of the chromosomes until mitosis, resulting in the formation of non-replicated heterochromatin bridges and breakage at anaphase. Rearrangements involving breakage preferably at or close to heterochromatin regions have been reported by several workers (Murata and Orton, 1984; Johnson et al., 1987; Lee and Phillips, 1987; Benzion and Phillips, 1988).

A unique case of structural variation has been reported in triploid ($2n = 3x = 18$) *Scilla siberica* (Deumling and Clermont, 1989). Extensive chromatin dimunition during callus culture resulted in chromosomes of about one-tenth the original size. Plants regenerated from the callus possessed only 20–30% of the original DNA per cell although showing significantly increased chromosome number ($2n = 30–40$).

9.5.3. Gene mutations

In recent years several somaclonal variants due to recessive or dominant single or multiple gene mutations have been described. Recessive mutations may not express in the R_0 generation of somaclones but can be detected in their selfed progeny.

Through conventional genetic complementation tests of several tomato plants regenerated from leaf callus, 13 distinct gene mutations have been well characterized and mapped to specific loci on the chromosomes (Evans and Sharp, 1983, 1986; Evans and Bravo, 1986). The yellow fruit (chromosome 31), orange fruit (chromosome 10), jointless pedicel (chromosome 11) were single recessive gene mutations while the *Fusarium* Race 2 wilt resistance (chromosome 11) was a single dominant gene mutation. Somaclones showing single gene recessive mutations are also reported for rice (Fukui, 1983; Sun et al., 1983), tobacco (Prat, 1983) and maize (Edallo et al., 1981). In red clover, variations were due to additive effects of mutated genes (Keyes et al., 1980).

9.5.4. Gene amplification

Brown (1981) suggested that any gene that cannot modulate its expression is likely to show gene amplification, if the right selection agent is available. By subculturing alfalfa suspension in the presence of the herbicide phosphinothricin, Donn et al. (1984) selected lines showing 20- to 100-fold more resistance to the herbicide than the wild type. The resistance was caused by a 4–11-fold amplification of glutamine synthase (GS) gene and a consequential 3–7-fold increase in GS enzyme. This herbicide is a competitive inhibitor of GS. Goldsbrough et al. (1990) selected *Nicotiana tabacum* cell lines resistant to normally lethal concentrations of glyphosate and found that the level of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which is inhibited by glyphosate, was elevated. Increased mRNA levels for EPSPS resulted from the amplification of at least two genes encoding this enzyme; the amplification increased with the level of glyphosate. The selected cells maintained the elevated EPSPS mRNA level even in the absence of the herbicide, suggesting that selection resulted in stable genetic modification.

9.5.5. Changes in extranuclear genes

The genetic material of cytoplasmic organelles can also be altered by in vitro culture conditions (Brown, 1991; Kane et al., 1992). Higher plant mitochondrial DNA is usually regarded as a circular molecule, containing repeated sequences which act as sites for intragenomic recombination (Palmer and Shields, 1984). Many conformational and molecular changes in mitochondria in response to tissue culture have been observed. Tobacco suspension cultures showed variability in mtDNA restriction fragment pattern. In these cases most of the mtDNA was organized as amplified circular DNA molecules derived from the main mitochondrial genome (Hanson, 1984; Newton, 1988). Hartman et al. (1989) demonstrated significant alterations in the mtDNA of wheat plants regenerated from tissue culture; the extent of change was determined by the length of the tissue culture period. The non-embryogenic calli and non-embryogenic sectors of embryogenic calli of wheat suffered a loss of about 8 kb fragment from the mtDNA found in the embryogenic cells (Rode et al., 1988). In maize, five of the six male fertile, resistant mutants obtained from male sterile populations susceptible to specific toxin of *Drechslera maydis* race T were characterized by a change in about 6000 base pairs in the mtDNA (Gengenbach and Connelly, 1981; Kemble et al., 1982; Umbeck and Gengenbach, 1983).

The chloroplast genome is relatively more conserved. One of the most frequent and noticeable manifestations of somaclonal and gametoclonal variation in grasses is the production of albino plants. Day and Ellis (1985) showed that the albino plants from anther cultures of rice may have lost up to 70% of the plastid genome. Dunford and Walden (1991) analysed five albino barley plants regenerated from microspores by direct embryogenesis. Four of the plants had undergone deletion or alterations in specific restriction fragment of the chloroplast genome. Sun et al. (1979) found that androgenic albino plants of rice contained little or no 16S or 23S RNA as well as significantly reduced levels of Rubisco protein. Since chloroplast rDNA is encoded by chloroplast DNA, any reduction in chloroplast rDNA should be due to impairment of cpDNA.

The emergence of low temperature resistant plants in anther cultures of temperature-sensitive line of *Nicotiana tabacum* has been shown to be due to changes in the genetic factors of the cytoplasmic organelles (Matzinger and Burk, 1984).

9.5.6. Activation of transposable elements

Transposable elements are genetic sequences which are capable of moving around in the genome and modify gene expression. A number of such elements (e.g. Ds, Ac, Mu) have been described in maize. Insertion of such elements at a new locus causes unstable suppression or modification of the transcriptional property of adjacent genes. Excision of the element may allow reversion to the wild phenotype. The excision is affected by several factors such as virus infection, temperature, chromosome breakage and the genetic background (Stafford, 1991).

McClintock (1984) had suggested that genomic shock can trigger transposition of these elements. There is growing evidence to suggest that the stress of tissue culture conditions can also activate previously silent transposable elements (Groose and Bingham, 1986a; Lee and Phillips, 1988; Phillips et al., 1990).

Activation of maize transposable elements following tissue culture has been reported more than once. The maize plants regenerated from tissue cultures were found to contain an active *Ac* element whereas none had been detected in the initial explant (Evola et al., 1985; Peschke et al., 1987; Phillips et al., 1990). Similarly, Evola et al. (1984) observed the activation of an *Spn* (*En*) element in half of the regenerated plants of maize. More than 20% of the alfalfa plants regenerated from tissue cultures of a white flowered somaclone exhibited the wild type purple flowered phenotype (Groose and Bingham, 1986a,b). Genetic analysis indi-

cated that while the wild type and mutated alleles were stable and sexually transmitted the culture process appeared to trigger reversion, suggesting the involvement of transposable elements.

9.5.7. DNA methylation

Phillips et al. (1990) have proposed the hypothesis that most, if not all, tissue culture-induced mutations are directly or indirectly related to alteration in the state of DNA methylation. The degree of DNA methylation of genes is inversely co-related with their expression. Accordingly, increase or decrease in DNA methylation might account for qualitative mutations, such as those controlled by single recessive genes, for increased transposable element activity, for simultaneous changes in qualitative characters and the mutations caused by chromosome bridges.

In two experiments involving *Ac* (Activator) elements, which cycle between activity and inactivity at a relatively high frequency, the elements were fully methylated when inactive but less methylated when they regained activity (Schwartz and Dennis, 1986; Chomet et al., 1987). One of these cycling with *Ac* elements put into tissue culture in inactive form showed activation of the element at 80× the normal rate for the allele (Dennis and Brettle, 1990). This increased activity was correlated with reduced methylation in the 5′ end of the element. Some correlation between tissue culture derived *Ac* genetic activity and reduced methylation of *Ac* homologous sequences has also been detected by Peschke et al. (cited in Phillips et al., 1990). Several other authors have described significant alteration in methylation status of plants regenerated from tissue cultures of maize (Brown, 1989, 1991), tobacco (Durante et al., 1989) and carrot (Palmgren et al., 1991). In maize and many other cereals the altered methylation status was stably inherited (Brown, 1991).

The occurrence of quantitative variation with extremely high frequencies in the plants regenerated from tissue cultures of maize inbred lines, led Phillips et al. (1990) to suggest the involvement of highly mutable, yet heritable DNA methylation.

9.6. ASSESSMENT OF SOMACLONAL VARIATION

The most useful somaclone would be one which conserves all the good characters of the parent cultivar with the addition of a specific desirable trait in which it was lacking. It is, therefore, extremely important to select the variants as early as possible, with minimum exposure of cells to

tissue culture environment. With prolonged culture gross abnormalities may appear (Bhojwani and Arora, 1992). While most of the deleterious changes are sieved during regeneration and field transplantation of the in vitro raised plants, some abnormalities do persist. Early detection and rejection of variants is also desirable in order to reduce losses in micropropagation.

Somaclonal variants have been mostly assessed at the phenotypic level, and in over 50% cases it is based on R_0 plants. Phenotypic screening of R_0 plants would allow detection of only dominant or homozygous traits. Moreover, the possession of normal phenotype by R_0 plants does not reflect a lack of variation. Recessive mutations in heterozygous regenerants can be recognized only in their segregating R_1 and R_2 progenies. It is, therefore, important that the variants should be assessed in the sexual progenies of the in vitro regenerated plants so that their heritability is established.

The extent of somaclonal variation at the phenotypic level is usually determined as the percentage of plants showing aberrations for one or more defined characteristics, such as plant height, heading date, tiller number, time of flowering, fertility, flower and fruit colour, yield, tolerance to salinity, disease resistance, etc. De Klerk et al. (1990) have suggested that the degree of variation in somaclonal population may be assessed by determining the value of standard deviation (SD) for a given quantitative trait. Since somaclonal variation renders the genotype more heterozygous the selfed progeny of the regenerants are expected to show increased SD for various quantitative traits (Jackson and Dale, 1989). The advantages of quantitative SD-assay over the qualitative assays are: (1) for each determination the number of plants needed is smaller (20–30 instead of 100); (2) the observation of these parameters does not depend upon the eye of the observer; (3) in a short period of time many individuals can be evaluated; and (4) the SD assay system seems to be much more sensitive.

As the main interest in somaclonal variation is a novel source of variability in plant breeding, most identifications of somaclones have been done on the basis of gross morphological features. Where this was not possible biochemical characterization was performed, usually involving protein electrophoresis. Both these techniques assess the phenotype of the plant which is affected by the environment. Assessment of genotype of the plant is a more rapid and precise method of assessing somaclonal variation. In this regard much effort has been placed on chromosome analysis (Geier, 1991). However, gross changes in chromosome number and morphology cannot account for all the observed variations at the whole plant level. RFLP offers a better method to analyse somaclones,

both from the point of view of identifying subtle changes and also in the ability to analyse plants from different environments, even while still in culture. Several reviews on the impact of this technique in plant breeding have been written (Beckman and Soller, 1986; Tanksley et al., 1989; DeVerna and Alpert, 1990). RFLP analysis for somaclonal variation assessment aims at identification of altered band pattern instead of selecting the presence or absence of alleles at different loci. For details see Potter (1991).

9.7. PRACTICAL ASPECTS

Somaclonal variation provides an additional source of genetic variability suitable for exploitation in crop improvement programmes. It is appealing to breeders because it occurs at a frequency often considerably higher than the incidence of spontaneous mutations or chemically induced mutations (Gavazzi et al., 1987; Lindsey and Jones, 1990; Phillips et al., 1994) and, thus, accelerates the process of selecting desirable genotype over the classical breeding approaches based on sexual hybridization. During tissue culture of maize the frequency of nucleotide substitution in the Adh1-1s allele was found to be 10 000 times higher than the spontaneous mutation rate (Dennis et al., 1987). Similarly, up to 15% variants were detected in regenerants of tomato against a spontaneous mutation rate of 1×10^{-6} (Evans and Sharp, 1986). Sun et al. (1983) reported 72% variants in rice regenerants. Sometimes unique mutations have been generated through tissue culture which could not be obtained through crossing or mutagenesis (Gavazzi et al., 1987; Semal and Lepoivre, 1990). For example, in potato introduction of traits such as higher yield, low colonization by aphids without disturbing the other characteristics of the parent cultivar is unique to tissue culture derived variability (Semal and Lepoivre, 1990).

The variability generated from tissue culture is already being exploited, and at least ten new varieties have been released based on somaclonal variant selection (Table 9.2). Vegisnax, comprising fresh pre-cut 'ready-to-eat celery and carrot sticks', introduced in the US market, is another example of successful commercial exploitation of somaclonal variants (Lindsey and Jones, 1990). In Hungary a somaclone variety of rice 'DAMA' has been recently released (Heszky and Simmon-Kiss, 1992). Among the existing rice varieties of that country, DAMA is most resistant to *Pyricularia* and has the best seed profile and cooking quality. DNA Plant Technology Corp., USA, developed a seedless bell pepper cv 'Bellsweet' from doubled haploids of the var. 'Golden cal wonder' produced by anther culture (Morrison and Evans, 1988). A few somaclones of

fennel obtained through somatic embryogenesis have been used in conventional breeding programmes (Desmaret et al., 1987).

As single gene mutations and organelle gene mutations have been observed in somaclonal variants, an obvious strategy to produce new cultivars through tissue culture is to introduce the best available cultivar into tissue culture and select for improvement of a specific trait amongst the regenerated plants. Hence somaclonal variation could be used to uncover new genotypes that retain all favourable characters of the existing cultivar while adding one additional trait. This approach is particularly attractive when breeding by classical sexual crossing is hampered by the lack of genetic variability to generate new hybrid classes or by lengthy hybridization and selection processes necessary to obtain a variety with new desirable traits. Potato is one such example.

On the negative side, the occurrence of uncontrolled somaclonal variation in tissue culture raised plants is at present limiting the use of in vitro methods for clonal propagation of selected genotypes and commercial production of industrial compounds from cell cultures. In the Netherlands alone losses of over US$1 million per year have occurred due to somaclonal variation (De Klerk, 1990). In most micropropagated crops somaclonal variation is avoided by adopting the axillary branching approach to shoot multiplication (see Chapter 16). However, even in these cultures, adventitious buds are often formed without notice and introduce variation which is multiplied in the subsequent cycles of shoot-proliferation (Marcotrigiano et al., 1987). In standard in vitro proliferation of chimeral strawberry, adventitious buds account for 80% of the newly formed shoots, a percentage similar to the amount of off-types (rearranged chimeras) obtained (Marcotrigiano et al., 1987). Rarely, the plants raised from the cultures of apical or lateral meristems also exhibit variation (Moyer and Collins, 1983). Elimination of cryptic or latent virus in meristem cultures may be an important cause of these variations. In addition, micropropagation via somatic embryogenesis in liquid medium, which can be automated, is a cheap alternative for the present day methods; it is hampered by, among other things, somaclonal variation. Somaclonal variation is also a potential threat for the production of genetically engineered plants without loss of any useful trait of the parent cultivar, as it involves a tissue culture step. Somaclonal variation also affects the production of secondary plant substances by cell cultures, thereby reducing the production by well established cultures (Deus-Neumann and Zenk, 1984).

Since in vitro regeneration of plants serves two diverse objectives, cloning and sub-cloning of plants, it is important to select the appropriate in vitro approach best suited to the objective.

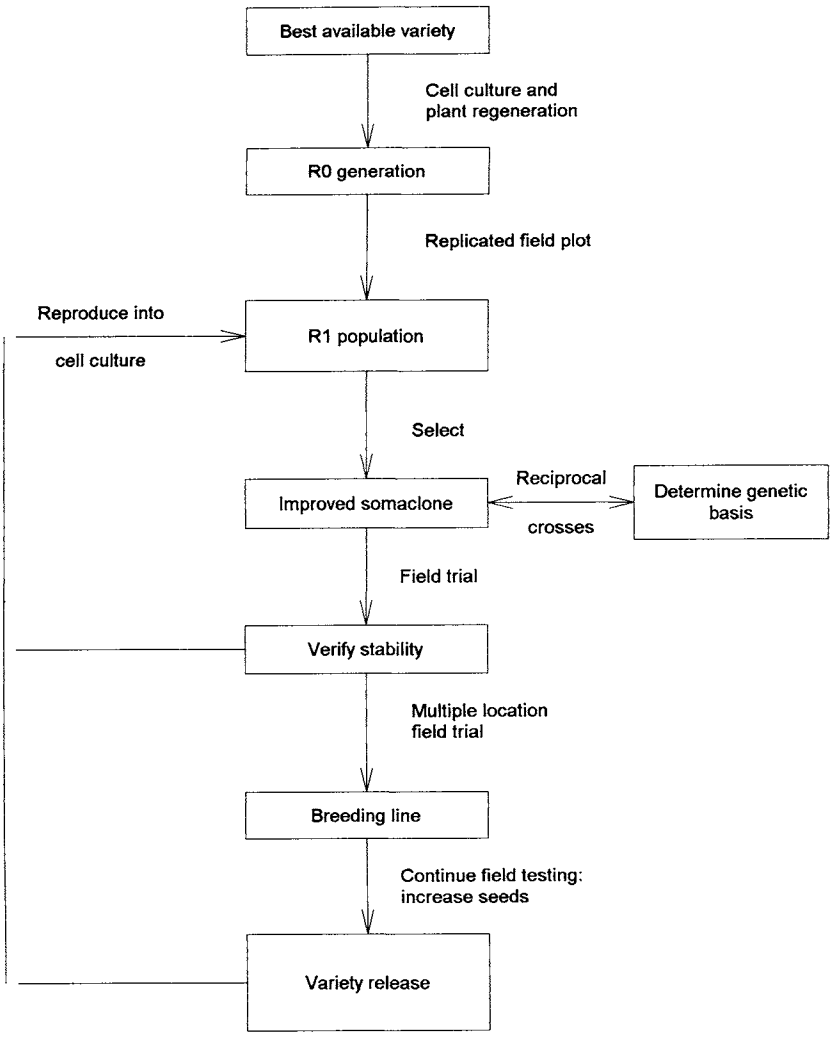

Fig. 9.9. A strategy for the production of somaclonal variants to develop new varieties (adapted from Evans et al., 1984).

9.8. CONCLUDING REMARKS

Tissue culture conditions induce a range of genetic changes, ranging from numerical and structural changes in the chromosomes to gene mutations, gene expression, and gene amplification, in cultured cells and the plants regenerated from them. Consequently, as stated by Phillips et al.

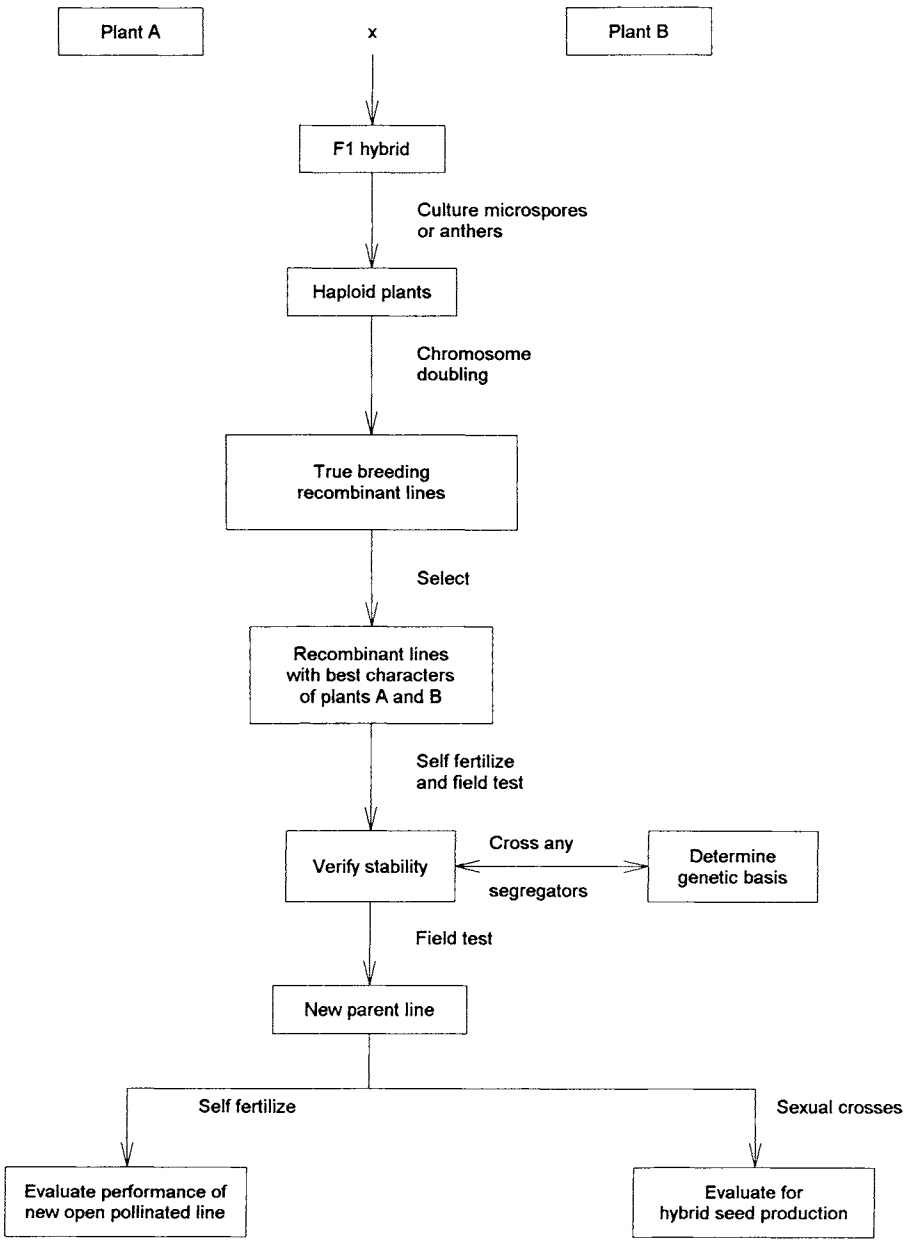

Fig. 9.10. A strategy for the production of gametoclonal variants to develop new varieties (adapted from Evans et al., 1984).

(1994), 'no two of the callus-derived plants are exactly alike and, none is just like the plant that donated the cell or cells for tissue culture'. This is proving to be a serious limitation in exploitation of the full potential of in vitro techniques for clonal plant propagation. Even when the most conservative approach of axillary bud proliferation is followed to clone plants, some adventitious buds may develop and introduce genetic variation. Therefore, to avoid variation in tissue cultures would be highly desirable. Pretreatment of leaf tissue of haploid *Petunia* with 30–100 μM BAP for 9–12 days prior to culture on regeneration medium had a stabilizing effect on the genome (Liscum and Hangarter, 1991).

Spontaneous variations generated in tissue cultures of somatic and gametic cells are being used as a novel source of heritable genetic variation suitable for upgrading the existing cultivars of crop plants. Breeders are finding it attractive because of its simplicity. During the past decade several somaclonal and gametoclonal variants have been utilized to release new varieties (Table 9.2) and some products are already in the market. Although some of the variants have been used directly as new cultivars, these variants may be more useful as breeding lines.

Since most of the mutations are recessive they cannot be detected in the R_0 somaclones, and require progeny analysis for unmasking. In this regard gametoclonal variants are more useful as they allow uncovering a recessive trait and additive characters with relatively small population. Figures 9.9 and 9.10 present breeding strategies for the use of somaclonal and gametoclonal variations for the development of new varieties.