Applications of tissue culture to the genetic improvement of grapevines

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Summary: The grapevine was among the first plants to be cultured *in vitro* (1944). Regeneration by somatic embryogenesis and organogenesis was reported in the 1970s and plantlet production from cell suspensions or callus is now a routine procedure in many laboratories. Methods for isolating grapevine protoplasts have yet to be achieved. The fragmented apex technique, involving high-frequency adventitious bud formation, is a novel and efficient method for rapid multiplication of grapevines but culture of anthers and pollen has been generally unsuccessful. Micropropagation procedures for *vinifera* grapes, *Vitis* species and interspecific hybrids, including rootstocks, are all available. Seedless-seedless hybridization, involving embryo rescue in crosses with stenospermocarpic female parents, is of major significance in breeding seedless table grapes.

There has been substantial progress in protoplast cell, tissue and organ culture of grapevines, but this technology is still less well developed than with some other fruit crops (notably citrus and apples). So far, tissue culture has little impact on genetic improvement. Exploitation of somaclonal variation for clonal selection is an attractive option for premium wine cultivars. There is evidence of somaclonal variation *in vitro* but the usefulness of this random genetic variation in viticulture is still uncertain. To date, results of field trials with vines from somatic embryos have been disappointing. The grapevine is proving to be a difficult subject for *Agrobacterium*-mediated genetic transformation (*A. tumefaciens* and *A. rhizogenes*) and microprojectile technology is another option which is being investigated.

Key words: tissue culture, somaclonal variation, protoplast technology, genetic engineering, biotechnology, breeding, genetics, review.

Introduction

The wine industry is characterized by extreme conservatism in the cultivars used for winemaking. Most of the world's 9 million ha of vineyards are planted with traditional cultivars which have been perpetuated for centuries by vegetative propagation. The well-known cultivars of French viticulture, such as Cabernet Sauvignon, Pinot noir and Chardonnay, are all of Roman or pre-Roman origin (LEVADOUX 1956; RIVES 1971; BOUQUET 1982 a). The histories of the traditional cultivars of other European grape-growing countries are equally as long.

Quality in wine has become closely associated with the winemaking characteristics of a relatively short list of traditional cultivars. Further, the growing of these cultivars has become fixed by custom, or by law under the system of Appellation d'Origine Contrôlée in France and by similar legislation in Italy and Spain. The traditional cultivars of Europe are also predominant in viticulture in the new worlds of North and South America, Australia and South Africa.

So far, wine grape breeding has made little impact at the level of the scion. The breeding of new wine grapes is feasible in technical terms, but new cultivars with unfamiliar names and wines with unfamiliar flavors face a battle for acceptance in the market place. As a consequence of these special circumstances, clonal selection, the exploitation of variation within traditional cultivars, has become a widely-used procedure for the improvement of wine grapes.

Rootstock breeding has made a substantial contribution to viticulture. The phylloxera resistant rootstocks bred in the late 19th century (e. g. SO 4, St. George, A x R #1) represent the first and most successful example of biological control of an insect pest. In the last 40 years, several new rootstock cultivars have been bred which confer resistance to nematodes or to unfavorable soil conditions. Table grape and raisin production is not subject to the constraints which affect wine production. Consequently, plant breeding has made a more significant contribution than with wine grapes. Many new table grape cultivars have been released in recent years, particularly of seedless grapes.

The advent of tissue culture and genetic engineering, and the application of this technology to crop improvement, has much significance for viticulture. There are two main areas of interest (i) procedures which improve the efficiency of conventional breeding and (ii) applications of cell and tissue culture which augment genetic variation within existing genotypes, i. e. clonal variation. The former has a major role in the breeding of rootstocks, table grapes and raisin grapes. The latter is of particular importance for premium wine grapes because of the 'genetic straightjacket' within which this form of viticulture is constrained by tradition, legislation and the market place.

Progress in grapevine tissue culture

A prerequisite for the application of tissue culture to grapevine improvement is the availability of highly efficient methods for plant regeneration or plant propagation *in vitro*. Substantial progress has been made in recent years but tissue culture of grapes has had a long history.

The grapevine was among the first plants to be cultured *in vitro* (Morel 1944). Proliferation of callus and formation of adventitious roots were the subjects of several reports during the 1950s and 1960s, but the grapevine proved to be recalcitrant with respect to regeneration *in vitro*. Somatic embryogenesis was first reported by Mullins and Srinivasan in 1976, and organogenesis was reported in the same year by both Favre (1976) and Hirabayashi *et al.* (1976). These developments were pre-dated by the first report on cultivation of grapevine protoplasts (Skene 1974). Since then, there have been several publications on methodological factors affecting isolation, survival and division of grapevine protoplasts (Skene 1975; Brezeanu *et al.* 1982; Hasler *et al.* 1982, 1983; Bessis *et al.* 1985; Lebrun 1985; Wright 1985; De Filippis and Ziegler 1985; Yamakawa *et al.* 1985; Barbier and Bessis 1988), but plant regeneration has yet to be reported.

There have been approximately 50 publications on micropropagation of grapevines since the original report of Jona and Webb (1978), i. e. reports on methods for induction of axillary shoot proliferation and subsequent formation of adventitious roots by microcuttings.

A novel method for rapid multiplication *in vitro* using fragmented shoot apices was developed by Barlass and Skene (1978) and has since been much refined (Barlass and Skene 1980 a, 1980 b; Barlass *et al.* 1981). In this procedure adventitious buds are formed with very high frequency in the tissues produced by cultured leaf primordia. Recently, it has been shown that numerous adventitious buds can be induced on hypocotyl explants of somatic embryos of grape cultivars (Vilaplana and Mullins 1989).

There is a single report from China of haploid plantlet production in grapevines (Zou and Li 1981), but attempts elsewhere to obtain haploids by culture of anthers and pollen of *Vitis vinifera* have been unsuccessful. In many grapevine genotypes the connective of anthers is a highly regenerative tissue and it gives rise to somatic embryos with high frequency (Rajasekaran and Mullins 1979, 1983). Callus produced by cultured anthers may contain haploid metaphases or nuclei in which the DNA content is consistent with the haploid condition (1C-2C), but derivatives of these cells do not seem to participate in embryo formation; plants from anther callus are diploid and heterozygous (Rajasekaran and Mullins 1983). Classical androgenesis involving internal divisions in pollen grains and extrusion of embryogenic callus, as seen in many Solanaceae and Cruciferae, has not been observed in grapevines. At the level of the intact plant, mixoploidy was observed in twinned seeds by Bouquet (1982 b), but no haploid individuals were recovered.

Application of the embryo rescue technique to stenospermocarpic grapes has enabled 'seedless-seedless' hybridizations. With this technique 'seedless' genotypes can be used as both male and female parents because zygotic embryos are rescued before they abort. This greatly increases the frequency of seedless progeny (Spiegel-Roy *et al.* 1975, 1986; Ramming and Emershad 1982; Cain *et al.* 1983; Goldy and Amborn 1987; Goldy *et al.* 1988).

Practical applications: overview

So far, seedless-seedless hybridization has had the greatest impact of any aseptic method on grapevine improvement, and it represents a major improvement in the methodology for breeding seedless table grapes. Haploids, and homozygous diploids derived from them, would be particularly useful for grapevine breeding and for genetic studies. However, it is now 18 years since the first experiments on cultivation *in vitro* of grapevine anthers (MULLINS 1971) and haploids are still unavailable. In fact, evidence for the existence of grapevine haploids is equivocal and there is a suggestion that haploidy may be a lethal condition in the clonal cultivars of *Vitis vinifera* L. (RAJASEKARAN and MULLINS 1983).

The main application of micropropagation has been in the production of pathogen free stock. Tissue culture was first used for virus elimination in the 1960s (GALZY 1964) and it is now a standard procedure in clean stock programs. Recently, the fragmented apex technique has been used to produce grapevines which are free from infection by viroids (DURAN-VILA *et al.* 1988). The relative ease with which nodal explants of grapevine cultivars can be induced to proliferate axillary buds has led to the use of micropropagation as a vehicle for mutation breeding (REISCH *et al.* 1985; BARLASS 1986; KIM *et al.* 1986). However, the usefulness of induced mutation for grape cultivar improvement has yet to be established.

In terms of potential applications there is much interest in the possibility that tissue culture procedures may be used to create or amplify genetic variation within commercially important cultivars of wine grapes and, thereby, provide new raw material for clonal selection. These potential applications are founded on the processes of somatic embryogenesis, organogenesis, and on the exploitation of both random genetic variation and directed genetic change.

Somaclonal variation in wine grapes – reality or illusion ?

In many species, plants regenerated from callus, cells or protoplasts exhibit considerable variation in morphological and physiological attributes (LARKIN and SCOWCROFT 1981; REISCH 1983; EVANS *et al.* 1984). This variability arises from gross changes in chromosome numbers, or structure, or from more subtle changes in the nuclear DNA which occur during the tissue culture process. The random spontaneous genetic variation which arises during plantlet formation *in vitro* is termed 'somaclonal' variation. Somaclonal variants of sugar cane, potato, rice, wheat, barley and rape have been discovered which possess disease resistance and several other agronomically interesting characters (SEMAL 1986). There has been enthusiastic speculation on the potential of somaclonal variation in perennial plant breeding (DE WALD and MOORE 1987), but experience so far has been disappointing. In viticulture, however, there is still great interest in somaclonal variation because it could provide a means of augmenting clonal variation (MULLINS 1985; MAURO *et al.* 1986).

In addition to variation that arises as a consequence of the tissue culture procedures, there are other potential sources of genetic variation in long-established cultivars of vegetatively propagated plants which are dependent upon tissue culture for their expression. Many fruit cultivars arose as somatic mutations are chimeric in structure, and rearrangements in chimeric structure occur during plant regeneration *in vitro* (MCPHEETERS and SKIRVIN 1983; SKENE and BARLASS 1983). In addition, ancient clones such as the traditional cultivars of grapevines are likely to have accumulated a considerable load of mutations over the centuries and cell culture methods may provide the means by which this normally covert variation can be expressed.

Grapevines of most major cultivars and many hybrids have now been regenerated *in vitro* by somatic embryogenesis using nucellar tissues of unfertilized ovules (MULLINS and SRINIVASAN 1976) or the vegetative tissues of anthers (RAJASEKARAN and MULLINS 1983). Hundreds, if not thousands, of grapevines have been produced from somatic embryos by researchers in several

countries. Evidence of somaclonal variation has come primarily from research on genotypes which are highly regenerative *in vitro*, for example, Gloryvine, a *Vitis vinifera x Vitis rupestris* hybrid. Gloryvines raised from somatic embryos often exhibit abnormalities such as dwarfism and albinism. Leaf shape is normally a highly stable character in grapes and it is the basis of ampelography (GALET 1979), but plants produced *in vitro* often show marked variations in leaf shape, including differences in petiolar sinuses and lobation. These differences tend to be transient and may be similar in nature to the temporary variations which occur in thermotherapy (VALAT and RIVES 1973) or after micropropagation *in vitro* (CHANCELLIER and COSSIO 1988). In addition, Gloryvines raised from somatic embryos show variation in sex expression (RAJASEKARAN and MULLINS 1983), indicating, perhaps, change in a single gene (DOAZAN and RIVES 1967; NEGI and OLMO 1971; ANTCLIFF 1980). Gloryvine is a male genotype, but 3 vines among the 125 which were planted in a field trial have proved to be hermaphrodite and they produced fruit for each of 5 years of the life of the trial.

In research on selection for salinity tolerance in *Vitis rupestris* SCHEELE cv. St. George (LEBRUN *et al.* 1985), cell lines were selected which grew in suspension cultures containing up to 150 mM NaCl. These apparently salt tolerant cell suspensions gave rise to somatic embryos, but the embryos became necrotic and died in the presence of 50 mM NaCl once radicle elongation had commended. From these results it appears that somaclonal variation in NaCl tolerance is manifested by cell suspensions of St. George but that tolerance at cellular level and in immature embryos is not closely correlated with tolerance in fully differentiated embryos and in intact plants.

In 1977, 12 vines of the original 'somatic Cabernet Sauvignon' (MULLINS and SRINIVASAN 1976) were planted at the Viticulture Research Station, Griffith, New South Wales, Australia. This planting was a curiosity rather than an experiment but it has provided some interesting observations. Initially, the somatic vines were highly variable in growth and cropping, as are most newly established grapevines, but they have become more uniform with the passage of time. A similar loss of variability with increasing age has been found in other grapevines regenerated from cells, for example, reversion to the ampelographically accepted leaf shape of the cultivar. The original 12 somatic Cabernet Sauvignon are now vigorous, well-established vines and are characteristic of their cultivar, but in other respects they are unremarkable.

A more extensive field trial was planted at Griffith in 1983 to compare the growth and cropping of Cabernet Sauvignon vines from somatic embryos and hardwood cuttings. Griffith is located in a hot, inland region of irrigated viticulture (Murrumbidgee Irrigation Area). The region is isolated and is phylloxera free. Grapevines are grown on their own roots. In this trial the somatic vines were juvenile at the time of planting and they have proved to be very slow to come into bearing. In the 1988 season, yield, fruitfulness (bunches/shoot), bunch size and trunk diameter were all substantially less than that of conventionally propagated vines. These results are difficult to interpret because it is not clear if the poor performance of somatic vines relative to the controls is related to juvenility effects or to an inherent inferiority. This can be overcome by re-propagating cuttings from bearing mother plants of the two types – somatic and conventional – and by establishing a new trial. It will be some years before the viticultural value of somatic embryogenesis can be determined.

So far, studies on somaclonal variation in wine grapes have given inconsequential, equivocal or disappointing results, but it is premature to conclude that this source of variation has nothing to offer to grapevine improvement because the mode of plant regeneration *in vitro*, somatic embryogenesis, may be unsuitable for the proper expression of somaclonal variation. In citrus it is now clear that somatic embryogenesis produces uniform propagules, including plants regnerated from protoplasts (VARDI *et al.* 1982; KOBAYASHI 1987). In many species, variability is most pronounced in populations derived from callus by organogenesis (VASIL 1983). In the case of grapevines, somatic embryo formation is preceded by a callus and cell suspension phase, but it seems that callus formation may be only one of the predisposing factors to the occurrence of

random genetic variation *in vitro*. Organogenesis from callus has been demonstrated in grapes but it occurs with low frequency (Favre 1976; Hirabayashi *et al.* 1976; Rajasekaran and Mullins 1981; Moriguchi *et al.* 1988). Refinement of these procedures to produce, on a routine basis, large populations of plants of the leading cultivars may provide access to levels of somaclonal variation that are useful for selection purposes.

The variation which arises in tissue culture, or which is induced by mutagens, is essentially random in nature and its successful exploitation is dependent upon the availability of rapid, accurate screening procedures. The development of these methods is relatively straightforward for characters such as disease resistance. Micropathogenicity tests are already available for selection *in vitro* for resistance to downy mildew (*Plasmopara viticola*: Morel 1948; Lee and Wicks 1982) and powdery mildew (*Uncinula necator*: Klempka *et al.* 1984) and selection at the level of phytoalexin production is an interesting possibility (Stein and Hoos 1984). It must be emphasized, however, that selection among somaclones for qualitative characters such as wine quality will remain as difficult and as time-consuming as conventional clonal selection with conventionally propagated grapevines.

Protoplast technoloy

The role of protoplast technology in plant improvement is to increase genetic variation. First, plants regenerated from protoplasts may exhibit somaclonal variation for agronomically useful characters. Second, by fusion of protoplasts it is possible to effect organelle transfer (chloroplasts and mitochondria) and gene transfer between sexually incompatible parents. Finally, protoplasts are useful in biotechnology for genetic transformation by direct uptake of foreign DNA or through procedures such as electroporation. However, the first step in applying protoplast technology to grapevine improvement is the availability of methods for plant regeneration from protoplasts. As indicated above, this has yet to be achieved but some progress has been made with isolation techniques.

Meanwhile, substantial advances have been made with other woody perennial fruit plants. Intergeneric hybrids have been produced by fusion of protoplasts from the sexually incompatible species *Citrus sinensis* and *Severinia disticha* (Grosser *et al.* 1988 b) and from fusion of protoplasts of pear and cherry (*Pyrus communis* var. *pyrasta* and *Prunus avium x P. pseudocerasus*: Ochatt *et al.* 1988 b). In addition, plants have been produced from fusion of protoplasts of sexually compatible species (*Citrus sinensis x Poncirus trifoliata*: Grosser *et al.* 1988 a). Plant production from protoplasts of individual species of *Citrus*, *Prunus* and *Pyrus* is now a routine procedure (Vardi *et al.* 1982; Ochatt and Power 1988 a, 1988 b, 1986; Ochatt *et al.* 1988 a). Thus far, callus has been produced by grape protoplasts (Skene 1975), but organ and plant regeneration has proved to be elusive. It is probable that lack of success with grapevine protoplasts reflects a lack of research, and a lack of researchers, in the field of cell biology of grapevines, and it is predictable that this technical blockage will be overcome in the near future. Judgment on the usefulness of protoplast technology for grape improvement must be reserved for the time being.

Genetic engineering

A prospect of biotechnology is that it may be possible to insert foreign genes into the genomes of traditional cultivars such as Cabernet Sauvignon and Chardonnay without altering the cultivars concerned in any of their other characteristics – including wine quality. Of special interest is the conferring of resistance to virus disease by incorporation of viral coat protein genes (Abel *et al.* 1986; Beachy *et al.* 1987; Cuozzo *et al.* 1988) or by expression of virus satellite RNA (Harrison *et al.* 1987). Another interesting possibility is the conferring of resistance to lepidopteran pests by

incorporation into the grapevine genome of genes encoding production of *Bacillus thuringensis* toxin (BARTON *et al.* 1987).

The current situation with the application of biotechnology to grapevine improvement is similar to that with protoplasts. Much has been written on the potential of biotechnology for genetic improvement of woody plants but the first step, the production of genetically transformed grapevines which express a marker gene, has yet to be reported. Genetically transformed grapevine roots have been obtained after inoculation of whole plants (cv. Grenache) grown *in vitro* (GUELLEC *et al.* 1988) with *Agrobacterium rhizogenes* containing two independent plasmids (i) the wild-type Ri-plasmid (pRi 15834) and (ii) a Tri-derived plasmid which carries the NPT II gene (neomycin phosphotransferase II) and the nopaline synthase gene. Expression of the NPT II gene confers kanamycin resistance to transformed plant cells. Recently, cell suspensions of Cabernet Sauvignon have been transformed by co-cultivation with *Agrobacterium* strains confirming resistance to kanamycin (BARIBAULT *et al.* 1989).

In some Australian work, plants exhibiting nopaline production and chimerism for kanamycin resistance were produced after co-cultivation of shoot apical fragments of grapevine cultivars with *Agrobacterium tumefaciens* containing the plasmid PGV3850::1103 neo (BENNETT 1988). However, the presence of foreign DNA could not be confirmed and attempts to purify these chimeric grapevines were unsuccessful. Microprojectile technology (MCCABE *et al.* 1988) is another approach to grapevine transformation which is being actively pursued. Microscopic particles of tungsten coated with DNA are literally 'shot' into the nuclei of meristems or regeneratively competent callus. The DNA concerned carries a marker gene, β-glucuronidase (GUS), which enables transformed cells to be identified by a color reaction (blue) when treated with the appropriate substrate.

So far, there have been encouraging preliminary results in several laboratories, both with *Agrobacterium*-mediated transformation and with particle acceleration, but no genetically transformed grapevines have emerged. This is in contrast to other horticultural crops such as pear (BROWNING *et al.* 1985), apple and strawberry (JAMES 1987), and walnut (DANDEKAR *et al.* 1988; MCGRANAHAN *et al.* 1988) where genetically transformed plants expressing marker genes have been reported. In the case of walnut, somatic embryogenesis was the means by which *Agrobacterium*-mediated transformation was achieved, and this route may yet be successful for grapevines. It is frustrating that the grapevine should prove to be such recalcitrant material for transformation when rapid advances are being made with other woody perennials. Sustained investment in research is needed if the exciting possibilities of biotechnology are to become realities in viticulture.

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