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REGENERATION OF GRAPEVINES BY ASEPTIC METHODS

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Abstract. Techniques are described for high-frequency somatic embryo and plantlet formation from (i) cell suspensions derived from nucellar callus of unfertilized ovules, and (ii) somatic cells of cultured anthers Plantlet regeneration by organogenesis, induction of adventitious buds and adventitious roots, has been achieved in a few genotypes of *Vitis* and in the Muscadine grape Factors affecting the regenerative competence *in vitro* of grape tissues include genotype (species, cultivar), growth phase (juvenile or adult), and origin of explants Competence is a heritable character Evidence is accumulating that grapevines produced *in vitro* are variable and that tissue culture *per se* leads to genetic variation It is concluded that the

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Abbreviations.
BA — benzyladenine (syn 6-benzylaminopurine)
2,4-D — 2,4-dichlorophenoxy acetic acid
NOA — β-naphthoxy acetic acid
PBA — 6-(benzylamino)-9-(2-tetrahydropyranyl)-9H-purine

future of tissue culture for grape propagation is uncertain but that aseptic methods hold great promise for grape improvement, especially in clonal selection

INTRODUCTION

Plant tissue culture techniques are important tools in programmes of crop improvement. Aseptic techniques are of special interest for use in horticulture and forestry where plant improvement by conventional methods is made difficult by heterozygosity and long generation intervals. Unfortunately the number of woody species which have been propagated *in vitro* by aseptic culture methods is small as compared with herbaceous species (11,17,18)

With the grapevine, the world's most widely-grown fruit crop, there has been much interest in sterile culture methods for plant propagation and improvement. In this paper we will present a summary of our earlier work (7,15) and give details of research in progress

MATERIALS AND METHODS

Plants of grapevine cultivars and species were propagated from cool-stored (4°C) cuttings by the method of Mullins and Rajesekaran (9). Seedlings were raised from stratified seeds (4°C for 8 weeks). Explants were surface sterilised by shaking with sodium hypochlorite (1% available chlorine) containing Tween-20 (0.1%) as a wetting agent. The basal medium of Nitsch and Nitsch (13) was used in all cultures.

Preliminary work showed that agar-based media were unsatisfactory for induction of organized development. However, agar-based media (0.8% w/v Difco agar) proved to be ideal for growing embryos and for micropropagation.

All the cultures which were established for induction of embryogenesis and organogenesis were grown on a gyratory incubator (80 oscillations min^{-1}, stroke amplitude 3 cm; 2.5 Wm^{-2} irradiance; 27°C). The provision of low intensity light prevented the callus from becoming red due to the formation of anthocyanin pigment.

Further details of explants, culture conditions and of media constituents are given with results.

RESULTS AND DISCUSSION

1. Somatic embryos from unfertilized ovules of grapes

Somatic embryos were produced from cell suspensions derived from the nucellus of cultured unfertilised ovules of *Vitis vinifera* cultivars (Cabernet Sauvignon, Grenache) and

from the hybrid grape, Gloryvine (*V. vinifera* L. × *V. rupestris* Scheele.) The ovules were excised from flowers collected 4 to 7 days before anthesis Callus of nucellar origin was produced by culturing the ovule explants initially in Nitsch medium containing 5 μM 2,4-D, or 5 μM β-NOA plus 1 μM BA, and then in a medium supplemented with NOA (10 μM) plus BA (1 μM). Embryos were produced in large numbers when callus was transferred to basal medium from which auxin and cytokinin were omitted.

The propagation of grape cultivars from nucellus has interesting implications. In citrus and mango, nucellar seedlings produced *in vivo* are usually virus-symptomless, due perhaps to exclusion of virus particles by the lack of vascular contact between the nucellus and its contiguous tissues (5). Similarly, grapevines regenerated from isolated nucellus may well be virus-symptomless and this possibility is being investigated in the field.

2. Somatic embryos from cultured anthers of grapevine

Anthers containing uninucleate microspores (from flowers 2 to 3 mm long) were chilled at 4°C for 72 h before culture in Nitsch medium containing 2,4-D (5 μM) and BA (1 μM). After culture in darkness for 10 to 20 days, a yellow-white callus was produced from the anther wall and the scar of the filament. Later, this callus was dispersed to form a suspension containing single cells and cell aggregates, and the suspension was transferred to basal medium. Callus which had been grown in the dark was transferred to light at this stage. During the next 20 days adventive embryos were formed with high frequency — 2500 to 3000 embryos per 25 ml of liquid medium.

This procedure, which was originally developed with anthers of Gloryvine, has been applied to a wide range of grapevine species, hybrids and cultivars. Among the *Vitis* species which have been investigated, *V. rupestris* and *V. acerifolia* (Syn. *V. longii* have the greatest propensity to embryo formation This character seems to be heritable and the regenerative competence of anther-derived tissues is exhibited by most hybrids in which *V. rupestris* is a parent. Included are highly complex hybrids such as J.S 23-416, Villard Noir and Villard Blanc, in which the contribution of *V. rupestris* is very dilute. In the *V vinifera* grapes, Grenache is the only cultivar which has been induced to form large number of embryos.

In all the species and cultivars which have been tested, maleness, as distinct from hermaphroditism, was observed to be an important factor associated with high degree of regeneration *in vitro*. When male inflorescences of Gloryvine were

converted into hermaphrodites by treatment with PBA, according to the method of Negi and Olmo (12), the ability of anthers to form callus *in vitro* was lost.

In both the ovule and anther cultures a higher percentage of secondary embryo formation was observed.

3. Establishment of plantlets

For production of normal plantlets the somatic embryos required chilling (4°C) for 2 weeks and the chilling treatment was effective in breaking dormancy when applied at any stage of embryogeny. Grape seeds also require chilling for 90 days (5°-10°C) for production of normal seedlings. Vigorous plantlets from somatic embryos were propagated by microcuttings (1-2 nodes) and were grown on filter-paper bridges so as to facilitate subsequent transfer to non-sterile media. Later, these propagules were grown in the glasshouse (24 to 26°C) in a medium containing peat and perlite (1:3).

4. Organogenesis in internode explants of grapevine

Internode explants (ca. 3 mm in length, average fresh weight 8 mg) from vigorously growing 40-day-old seedlings were cultured either in Nitsch medium supplemented with BA (1 μM) and 2,4-D (5 μM) or in a mixture of 2,4-D (5 μM) and NOA (5 μM). Organogenesis was observed 40 days after culture. The most frequent type of regeneration in callus cultures was root formation. Bud formation occurred after 60 days only in cultures derived from seedlings of Muscadine grapes (*Vitis rotundifolia*), Gloryvine, and two hybrids, both of which had Gloryvine as the male parent. These adventitious buds were found to arise near the surface of the callus. Explants from mature vines of *V. vinifera* cultivars, or from clonal Gloryvine failed to produce buds. The formation of buds occurred only in internodes derived from seedlings.

5. Propagation and storage of grapevines by microcuttings and somatic embryos

Plantlets are normally propagated in our laboratory by culturing shoot tips or "microcuttings", stem segments bearing an axillary bud. By use of cytokinins (BA or PBA) at concentrations of 5-10 μM it is possible to induce the outgrowth of numerous axillary buds and thereby increase the rate of plant multiplication.

It has been assumed hitherto that excised meristems provide genetically stable material for rapid clonal propagation (2), and for the production of disease-free plants (3). Accordingly, much attention has been given to micropropagation techniques for preservation of grapevine genetic stocks. Morel (6) demonstrated that by culturing excised shoot apices and

nodal segments under carefully controlled conditions of nutrient and environment, germplasm stocks of grapevines could be maintained with a fraction of the time, space and cost required to maintain them in the field. He calculated that 800 different grape cultivars could be stored in culture in a space of about two square meters, and that from a single meristem culture more than 10 million cuttings could be produced within one year.

However, there is a serious flaw in these proposals in that it has yet to be confirmed by the relevant field trials that grapevines which are propagated *in vitro* by microcuttings retain the unique characteristics which distinguish the major wine grapes There is some suspicion, from virus eradication work in France, that vines which are propagated *in vitro* exhibit alterations in morphology (10). It is not year clear if these alterations represent permanent genetic change (1), or whether they are physiological in nature and are associated with reversion to the juvenile growth phase (8).

It is now firmly established that the propagation of plants from callus, either by organogenesis or somatic embryogenesis, leads to significant variation in the propagules so produced (4,14,16).

CONCLUSIONS

The biochemical nature of regenerative competence is unknown and no information is available which might account for differences in regenerative behaviour *in vitro* among *Vitis* species and cultivars and among the different tissues of the individual plant In practical terms, however, sufficient technical information is now available to enable aseptic culture techniques to be used for large-scale multiplication of grapevines. An important qualification is that the fidelity of reproduction of aseptic methods *in vitro* is either in doubt or known to be suspect. At this stage it seems that tissue culture has an uncertain future as a means of propagating grapevine cultivars. However, aseptic methods hold great promise for use in grapevine improvement, especially in the sphere of clonal selection.

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