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Ploidy stability in grapevines following long term storage *in vitro*

by

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Stabilität des Ploidiegrades bei Reben nach Langzeitlagerung von *in-vitro*-Kulturen

Zusammenfassung: Für die routinemäßige Überprüfung der Chromosomen von Reben aus *in-vitro*-Kultur wurde eine modifizierte Methode zur Herstellung von Wurzelspitzen-Quetschpräparaten angewandt. Die Ergebnisse bewiesen, daß Adventivknospen, die durch Kultur zerstückelter Triebspitzen gewonnen wurden, diploid waren. Ferner wurde durch Lagerung multipler Sproßkulturen von acht *Vitis*-Genotypen — bis zu 12 Monate bei 9,5 °C — der Ploidiegrad der daraus regenerierten Pflanzen nicht beeinflußt.

Key words: tissue culture, chromosome, storage, temperature, Vitis, variety of vine.

Introduction

The culture of fragmented shoot apices of grapevine (FSAC) is a rapid method for producing large numbers of multiple shoots from adventitious buds (BARLASS and SKENE 1978, 1980 a, 1980 b). *Inter alia,* the technique is suitable for micropropagation (SKENE and BARLASS 1980), and shoot cultures from fragmented apices also have been used for *in vitro* germplasm storage (SKENE and BARLASS 1983 a). Both of these applications have a high requirement for genetic stability, one aspect being the strict maintenance of the chromosome number representative of the genotype (SCOWCROFT 1984).

Whilst it is well known that the chromosome number of callus and other dedifferentiated explants may be subject to change during *in vitro* culture (D'AMATO 1978; BAYLISS 1980), our earlier findings indicated that the ploidy of adventitious Cabernet Sauvignon shoots (2n = 38) obtained through FSAC remained unchanged from the original material (BARLASS and SKENE 1978). The current study was initiated to investigate this point in greater detail, as well as to observe the effects of culture on ploidy of a range of genotypes, both at normal temperatures and at the reduced temperatures used for germplasm storage.

Routine observations on large numbers of mitotic figures were made possible by modifications to existing root squash techniques.

Materials and methods

Plant material

Multiple shoot cultures were prepared from fragmented shoot apices of the genotypes listed in the table by methods described previously (BARLASS and SKENE 1978, 1980 b; SKENE and BARLASS 1980). Cytochimeric genotypes were avoided, as it has been shown that FSAC can separate the component cell types (SKENE and BARLASS 1983 b).

Once cultures were well established on a modified Murashige-Skoog medium, they were maintained for periods up to 12 months (0, 3, 6, 9, 12 months) at 9.5 °C in a 15 h

Grapevines from *in vitro* storage: numbers sampled for chromosome counts
Reben aus *in-vitro*-Lagerung: Anzahl der Proben für die Chromosomenzählung

Genotype	Number examined		
	Plants from culture	Root tip squashes	Mitotic figures
Vitis vinifera			
cv. Cabernet Sauvignon	56	226	1 272
Cabernet Franc	12	47	220
Sultana	53	285	1 768
V. amurensis	19	134	684
V. berlandieri	15	98	476
V. champini			
cv. Ramsey	26	148	703
V. labrusca			
cv. Concord	15	68	343
V. berlandieri × *V. rupestris*			
hybrid R 99	19	105	598
Total	215	1 111	6 064

light, 9 h dark photoperiod (white fluorescent tubes, 15—30 $\mu E \cdot m^{-2} \cdot s^{-1}$). In some cases, cultures were stored in constant darkness at the same temperature. This work is outlined by SKENE and BARLASS (1983 a).

At the completion of cold storage, material was transferred to fresh media and placed in a culture room with a 26 °C day and 20 °C night (BARLASS and SKENE 1978) until active shoot growth resumed. Shoots were transferred to rooting medium, ultimately hardened-off and placed in pots in an air-conditioned glasshouse for further growth prior to chromosome examination. For comparison, potted plants obtained by conventional propagation of hardwood cuttings were also available.

Examination of chromosomes

Pretreatment: 2—3 d before tips were required, each plant was tapped from its pot, and then gently returned to the same pot. This disturbance created air spaces into which actively growing roots proliferated. Root tips were collected in the morning, rinsed and placed in a 10 mg/l solution of *o*-isopropyl-N-phenylcarbamate (IPC) for 2.5—3.0 h at room temperature. IPC is very effective in contracting and separating chromosomes of a wide range of plant species (STOREY and MANN 1967).

Fixation: Tips were rinsed in at least two changes of distilled water at 10 min intervals, before immersing in freshly prepared Carnoy fixative (3 parts abs. ethanol : 1 part glacial acetic acid) for 2—16 h. Tips were then transferred to 70 % ethanol and stored at 4 °C. In general, observations were made the following day.

Tissue softening: The tips were taken from 70 % ethanol to 40 % ethanol for 10 min, followed by two 5 min changes of water, then left in water for a further 15—20 min whilst fresh enzyme solution was being prepared. Tissue was softened in 2 % w/v Macerozyme R-10 (Yakult Biochemical Co. Ltd., Nishinomiya, Japan) at pH 6.0 for 2.5—3.0 h at ambient temperature. Approximately 1.5 ml solution was used

for each five root tips. After maceration, tips were rinsed once in water for 5 min before hydrolysing in 5N HCl for 30 min at ambient temperature. This served to further soften the tissue, clear the cytoplasm and allowed easy separation of cells into a single layer when squashed. Care was taken not to overhydrolyse the tissues, as this caused the cells to burst easily. Tips were again rinsed in two 5 min changes of water, and left in water prior to preparation of squashes.

Squash preparation: Each root tip was transferred to a drop of 45 % acetic acid on a precleaned microsope slide, and the root-cap, epidermis and outer cortex were removed with dissecting needles, leaving a 0.5—1.0 mm tip. After further dissection into small pieces, a coverslip was added, and the preparation squashed between filter papers until the desired flatness was achieved. When required, squashes were made permanent by freezing the slide from below with either a stream of compressed CO_2 or a block of dry ice to facilitate removal of the cover slip, followed by washing in two 5 min changes of absolute ethanol and immediately mounting in Euparal.

Microscopic observation: Preparations were usually not stained prior to examination by phase contrast microscopy. Whenever possible, squashes were prepared from five roots per plant, and at least five mitotic figures were examined in each squash, preferably from cells in metaphase. Chromosomes were counted, and cells scored for ploidy ($2n = 38$).

In several instances, squashes were also prepared from shoot apices or immature tendrils by techniques essentially the same as described.

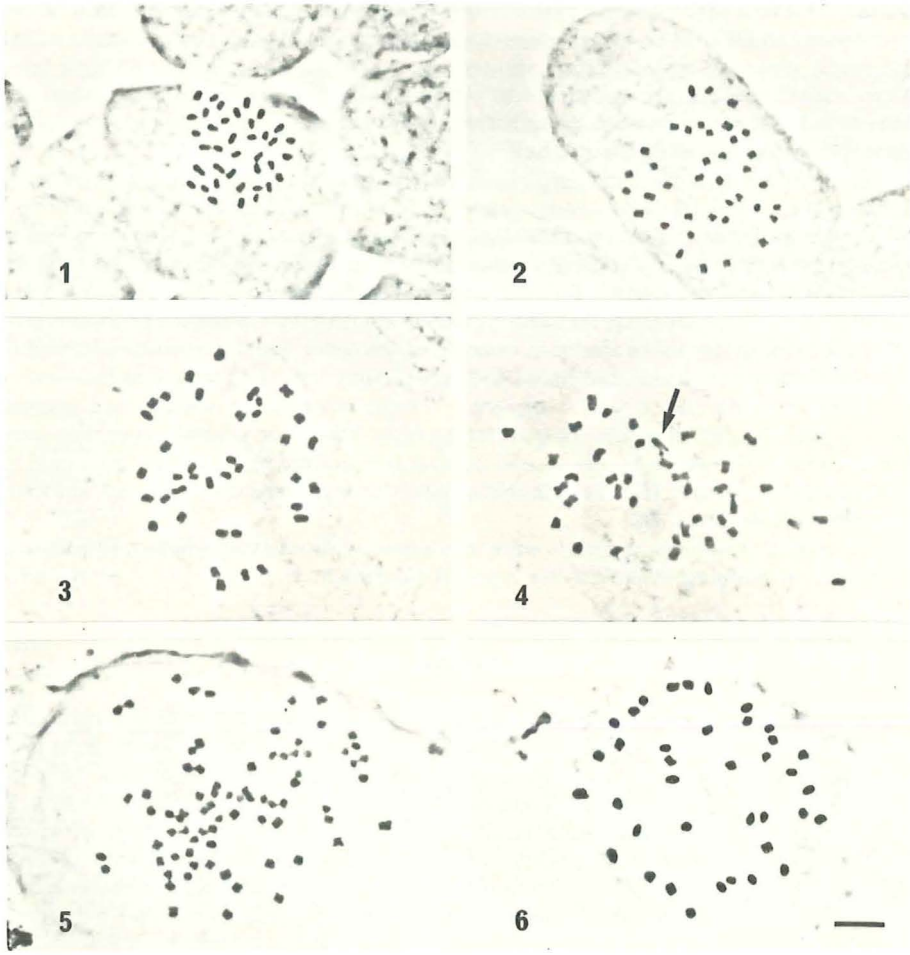
Results

In general, chromosomes were contracted, well separated, and confined within the boundaries of the cell (Figs. 1 and 2). If the cell had burst, cell separation in good preparations also was sufficient to avoid confusion in scoring chromosomes (Figs. 3 and 4). Contrast in density between chromosomes and background cytoplasm was good (Fig. 1) to excellent (Figs. 2—6), and occasionally chromosome detail was better than usual, with satellites being visible (Fig. 4). The examples illustrated represent cells of *Vitis vinifera* root tips taken from conventional cuttings (Fig. 1) and from tissue-cultured plants (Figs. 2—5). Squashes of similar quality were prepared from other genotypes (Fig. 6).

With the exception of Fig. 5 (a tetraploid cell from a mixoploid root with both diploid and tetraploid cells present), all examples are diploid. The majority of squashes were very flat, with chromosomes at the same plane of focus for photography and ease of counting.

Roots from 215 plants (8 genotypes) derived from tissue cultures were examined. This represented a total of 6064 mitotic figures from 1111 root squashes (Table). The diploid number of chromosomes was observed in roots from all plants. No aneuploid cells were detected in any of the preparations examined. Tetraploid cells ($4n = 76$) as well as diploid cells were present in root tips of 29 plants, and five other plants also bore both diploid roots and roots with tips appearing to be exclusively tetraploid. Shoot apices and tendrils were examined in all these latter plants and in selected plants with mixoploid roots; only diploid cells were observed.

For comparison, it should be noted that one Sultana vine in a series of glasshouse-grown plants derived from conventional hardwood cuttings also bore both diploid and exclusively tetraploid roots (thirteen 2n tips and five 4n tips, each from independent adventitious roots). As in the case of the tissue-cultured plants, only diploid cells were observed in the shoot apex.



Figs. 1—6: Metaphase plates of unstained grapevine root tip squashes observed by phase contrast microscopy. All at the same magnification. Bar (Fig. 6) = 5 µm.

Fig. 1: *Vitis vinifera* cv. Sultana, from conventional hardwood cutting. Diploid (2n = 38).

Figs. 2—4: *V. vinifera* cv. Sultana, *ex* tissue culture. Diploid. Arrow (Fig. 4) indicates chromosome with satellites.

Fig. 5: *V. vinifera* cv. Sultana, *ex* tissue culture. Tetraploid (4n = 76).

Fig. 6: *V. berlandieri* × *V. rupestris* hybrid R 99, *ex* tissue culture. Diploid.

Abb. 1—6: Metaphaseplatten in ungefärbten Quetschpräparaten von Rebwurzelspitzen, Phasenkontrastmikroskopie. Alle Abbildungen in gleicher Vergrößerung, Maßstab (Abb. 6) = 5µm.

Abb. 1: *Vitis vinifera* cv. Sultana, konventionell vermehrter Schnittholzsteckling. Diploid (2n = 38).

Abb. 2—4: *V. vinifera* cv. Sultana, aus *in-vitro*-Kultur. Diploid. Der Pfeil (Abb. 4) weist auf ein Satellitenchromosom hin.

Abb. 5: *V. vinifera* cv. Sultana, aus *in-vitro* Kultur. Tetraploid (4n = 76).

Abb. 6: Kreuzung R 99 (*V. berlandieri* × *V. rupestris*), aus *in-vitro*-Kultur. Diploid.

No attempt has been made to provide detailed results for individual experiments, as all plants coming from culture were considered diploid (as confirmed by examination of shoot apices where necessary), and the occurrence of mixoploid or tetraploid roots was independent of either culture temperature or the length of time stored at reduced temperature in light or darkness.

Discussion

Grapevine chromosomes are difficult to count due to their number and small size, a situation exacerbated by dense cytoplasm, which does not permit easy separation of chromosomes during preparation of squashes. Nevertheless, for routine counting of chromosomes in large numbers, reliable methods for their visualisation are needed. There are several reports of the examination of grape chromosomes (e. g., THOMPSON and OLMO 1963; RAJ and SEETHAIAH 1969; BOUQUET 1978), and some have produced good results. During this study, however, results with published methods were inconsistent, especially with respect to flatness of the figures, the degree of staining and amount of contrast between chromosomes and background cytoplasm.

The method of squash preparation finally adopted permitted the routine examination of large numbers of root tips. IPC proved to be particularly effective in contracting grape chromosomes, and combined with a careful balance between Macerozyme and HCl, the tissue was softened sufficiently to obtain consistently flat figures with good chromosome separation. High contrast between chromosomes and background was achieved by the clearing effects of fixative and HCl on cytoplasm, and especially by the simple expedient of examining unstained material with phase contrast microscopy. In this way the task of counting the small grape chromosomes was relatively simple.

The examination of root squashes from *in vitro*-cultured plants in this study has presented a great deal more evidence in support of previous conclusions based on Cabernet Sauvignon, that plants developed from adventitious buds following FSAC retained the diploid number of chromosomes (BARLASS and SKENE 1978). In particular, chromosome numbers of eight genotypes remained unchanged during extended culture at normal temperatures (26/20 °C) and were unaffected by storage at 9.5 °C for periods up to 12 months.

The presence of mixoploid meristems with diploid and tetraploid mitotic figures in roots from some of the plants is not unusual, as it is possible for polyploid mitoses to occur within the older parts of a predominantly diploid meristem (NAGL 1978). The occurrence of five plants with both tetraploid and diploid root tips is puzzling. They are unlikely to be cytochimeras, as shoot apices were exclusively diploid. In view of the plant propagated from hardwood cuttings (also with a diploid apex) that bore several tetraploid roots, it is suggested that these changes in root ploidy were a consequence of events occurring at the level of the root or its initials during growth in the glasshouse, and were unrelated to *in vitro* culture.

It is concluded that from the point of view of stability of chromosome number, FSAC is a suitable method of preparing multiple shoot cultures from non-chimeric *Vitis* species, and these may be stored at reduced temperature without change in ploidy level.

Summary

A modified method to prepare root tip squashes was used for the routine examination of chromosomes of grapevines developed from *in vitro* shoot cultures. The results

established that adventitious buds obtained by culture of fragmented shoot apices were diploid, and that storage at 9.5 °C of multiple shoot cultures of eight *Vitis* genotypes for periods up to 12 months did not affect the ploidy level of plants regenerated from them.

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