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Origin of triploid plants from anther culture of *Vitis vinifera* var. Grenache

by

Li Wei and Cao Ziyi

Agronomy Department, Gansu Agricultural University, Lanzhou, China

Summary: Microspore development in anther cultures of *Vitis vinifera* var. Grenache (2x) was observed with the PICCH staining method. There were five developing types: Hollow grain type, Standing type, Storage type, Free nuclei type and Coenospore type. Only microspores of the Coenospore type became multicellular. Most microspore types retrogressed during culture except the Hollow grain type. Plants developing from different anthers were mainly triploid (86.5 % of triploid cells). They seem to originate from karyomixis in microspores during anther culture.

Key words: Anther culture of grapevine, microspore in anther, triploid plant, karyomixis.

Introduction

Anther culture of grapevine was first reported by Mullins (1971). Gresshoff and Doy (1974) obtained haploid callus and Hirabayashi *et al.* (1976) regenerated plants from anthers. However, most regenerated plants were diploid (Cao *et al.* 1980; Hirabayashi and Akihama 1982; Rajasekaran and Mullins 1979, 1983; Bouquet *et al.* 1985; Stamp and Meredith 1988) except one regenerated from pollen (Zou and Li 1981). In anther cultures numerous nuclear irregularities have been described (Torrey 1976; Shimada 1971; Wu *et al.* 1980; Hu *et al.* 1980; Sunderland 1974). The origin of such plants regenerated from anther culture can not only be determined from the number of chromosomes. This paper describes microspore development and chromosome number in root tip cells of regenerated plants from the anthers of diploid *Vitis vinifera* var. Grenache.

Material and methods

In May 1989, 1990 and 1991 anthers from Grenache (2x) were inoculated on a B5-medium, supplemented with 4.0 ppm 6-BA and 1.0 ppm 2,4-D and cultured in total darkness at 25-28 °C. Every 10 d 30 anthers were taken randomly and fixed in ethanol (95 %)/chloroform/aceton (6:3:1) for 24 h. Then, the anthers were stained on a microscope slide with 1-2 drops PICCH for 30 min at room temperature and observed in squash preparations. Calluses were transferred onto fresh B5-medium and 30 d after inoculation cultured in continuous illumination (2000 lx) at 25-28 °C. After development of the first embryos, the light intensity was increased to 3000 lx. The first plants were observed 80 d after inoculation.

Analysis of ploidy level of regenerated plants was carried out 10-15 d after transfer of the plants to fresh nutrient medium. Root tips were excised at 10.00 a.m., stored in water at + 5 °C for 24 h and then fixed in (3:1) ethanol (95 %)/acetic acid for 24 h. After hydrolysis and maceration in HCl (37 %)/ethanol (98 %) (1:1) for 5-10 min at room temperature the root tips were stained with carbol fuchsin for 4 h and squashed in 1-2 drops of acetic acid (45 %). From every anther line, five regenerated plants were examined.

Correspondence to: Dr. Li Wei, Gansu Agricultural University, Agronomy Department, Lanzhou, People's Republic of China

Results and discussion

The development of microspores can be differentiated into five types:

1. Hollow grain type: Such microspores had contracted cell walls and were not stainable with PICCH (Fig. 2 b, left). Their proportion increased gradually during cultivation to about 70 % after 60 d (Fig. 1).

2. Standing type: These microspores showed a contraction around the germ pore and had weakly stained but well distributed cytoplasm (Fig. 2 a, left). Their development seemed to stop some time after inoculation and they seemed similar to the E pollen grains (embryogenic pollen grains) described by SUNDERLAND (1974). After 10 d of culture the highest percentage of 20 % microspores of this type could be determined (Fig. 1). With increasing culture time the intensity of staining was decreased due to contraction of the cell wall. Most of these microspores changed into hollow grains but some of them were stainable for some weeks.

3. Storage type: In microspores of this type many grains of different size and shape were observed (Fig. 2 a, middle and right). Their pollen wall was thicker than in other types. Staining with J/KJ showed some starch in the microspores. 10 d after inoculation 55 % of the microspores were of this type (Fig. 1) but as time went on they changed into hollow grain types or disintegrated and disappeared. This microspore type is typical for abortive pollen (NAKATA and TANAKA 1968; NITSCH 1970; ZHOU and YANG 1980).

4. Free nuclei type (maximum after 40 d): The cell walls of these microspores were thicker than those of the standing type (Fig. 2 b, right and a, left). They contained numerous well stainable grains which could be easily separated and might have been nuclei (Fig. 1). 40 d after inoculation 8.5 % of the microspores were of this type.

5. Coenospore type: Only two or three cells of this microspore type could be seen (Fig. 2 c, d). The thickness of their pollen walls was comparable to the standing type from which they may be derived. When the highest percentage (1.5 %) was measured 40 d after inoculation (Fig. 1) the callus developed continuously from the anther (Fig. 2 f). Many authors think that this type of microspores has a greater potential to develop callus (SUNDERLAND and DUNWELL 1977; ZHOU and YANG 1980).

Only a few microspores of the coenospore type developed and the number of microspores of the other types decreased during culture except the hollow grain type. Eventually a multicellular mass developed from the microspore (Fig. 2 e). 60 d after inoculation the first embryoids developed from anther callus (Fig. 2 f) and 80 d after inoculation the first plants appeared on the embryoids. Root tips of anther-derived plants contained 78-90 % triploid cells.

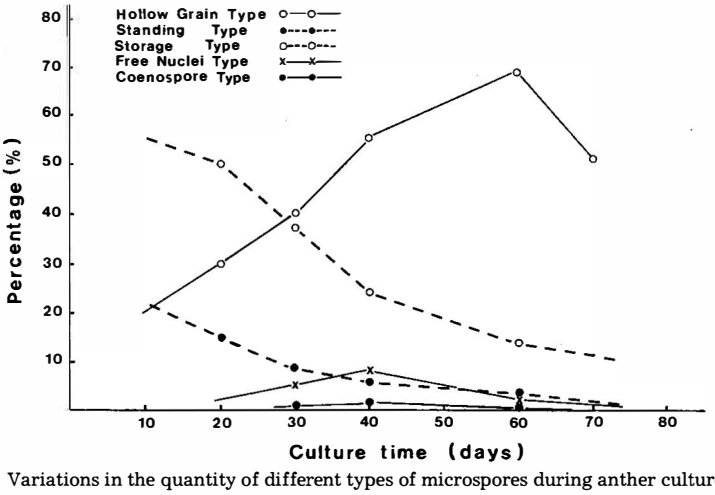

Fig. 1: Variations in the quantity of different types of microspores during anther culture.

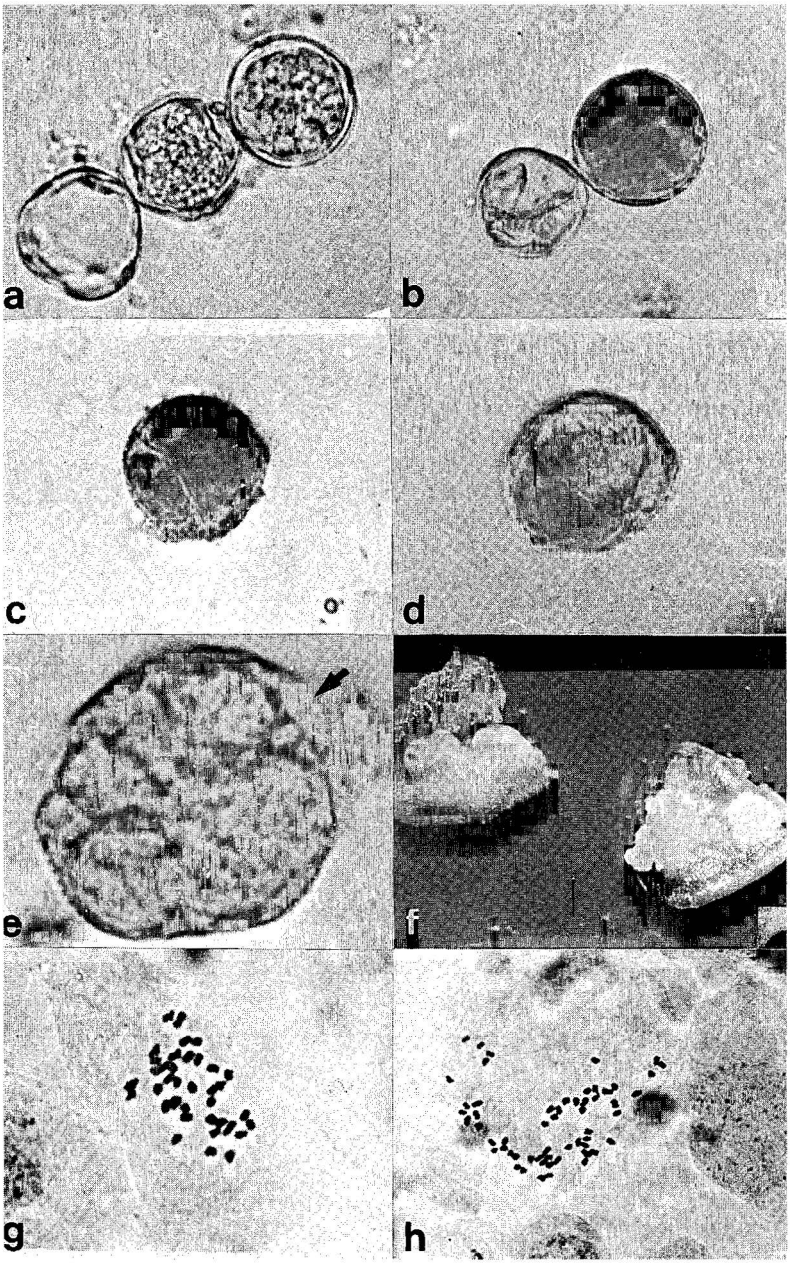

Fig. 2: Developing types of microspores during anther culture of Grenache (*Vitis vinifera* L.) and chromosomes of Grenache mother plant and the regenerated plant. a) Left side: Microspore of the standing type, 400 x; Middle and right side: Microspore of the storage type, 400 x; b) Left side: Microspore of the hollow grain type, 400 x; Right side: Microspore of the free nuclei type, 400 x; c) One microspore with two nuclei, 400 x; d) One microspore with three nuclei, 400 x; e) A multicellular mass from a microspore, the germ pores are indicated by the arrow, 1000 x; f) Callus from anthers of Grenache; g) Chromosomes of Grenache mother plant (root tip cell, 2n = 2x = 38); h) Chromosomes of regenerated plant (root tip cell, 2n = 3x = 57).

Although the ploidy level of plants regenerated from different anthers was very similar (Table) and they seemed to originate from somatic cells, it is not certain whether there are triploid tissue or cells in the anther of grapevine. Even then, the developing potential of diploid cells is greater than that of triploid cells and plants of different ploidy level should be obtained. Therefore it seems that triploid plants are derived from the microspores rather than from somatic cells.

There are different reports about triploid plants derived from anther cultures of diploid mother plants, e.g. of Petunia (RAQUIN and PILET 1972), and of *Datura innoxia* where haploid, diploid and triploid plants have been regenerated from anther culture. It is reported that the origin of the haploid plants were young flower buds (3.0 cm), the origin of diploid plants were mainly old flower buds (5.0 cm), and the origin of triploid and diploid plants were older flower buds (6.5 cm) (ENGVILD *et al.* 1972). Nearly all plants regenerated from the anthers of older flower buds (1.0-1.5 cm) were triploid (ENGVILD 1973). ENGVILD supposed such plants to originate from karyomixis in microspore during anther culture. There are many other descriptions of regenerated plants with different ploidy levels originating from karyomixis in microspores (NISHI and MITSUOKA 1969; NARYANASWAMY and CHAUDY 1971; NIIZEKI and OONO 1971; SUNDERLAND 1974; ZENG and OUYANG 1980; CEN *et al.* 1981; HU *et al.* 1982).

Diploid *Vitis vinifera* var. Grenache is a genotype which easily regenerates plants from anther culture, but earlier reports were on diploid plants, possibly from somatic cells in the anther (RAJASEKARAN and MULLINS 1983; BOUQUET *et al.* 1985; STAMP and MEREDITH 1988). Our plants obtained by anther culture of Grenache were triploid, possibly due to microspore regeneration. The fact that triploid plants can be obtained by conventional crossing of diploid with tetraploid plants shows that triploid cells can survive and regenerate, and there are indeed triploid cells in the diploid grapevine (Table). However, it seems impossible that all plants regenerated from different anthers were triploid even if there are some triploid cells and 3n microspore (COLLINS *et al.* 1974) which should have less potentiality of development than diploid cells and 2n microspores in anther of grapevine. The homozygous diploid embryoids, of course, could develop by endoreduplication and karyomixis of monokaryotic microspore

Table

Cell ploidy in root tips of a diploid mother plant and plants regenerated from different anthers of Grenache (*Vitis vinifera* L.)

cultivar or line		Total number of cells observed	Haploid and hypodiploid		Diploid 2n=38		Triploid 2n=3x=57	
			No.	%	No.	%	No.	%
Grenache		107	0	0	105	98.1	2	1.9
Anther line	G-1	212	3	1.4	20	9.4	189	89.2
	G-5	105	3	2.9	20	19.0	82	78.1
	G-8	77	1	1.3	7	9.1	69	89.6
	G-27	64	2	3.1	5	7.8	57	89.1

(SUNDERLAND 1974), and in older anthers with dikaryotic microspores the generative nucleus might well have changed into 2c nucleus by chromosome duplication due to its tendency to divide continuously. The triploid embryoid could develop from a karyomixis between a vegetative nucleus at 1c level and a generative nucleus at 2c level under the specific conditions of *in vitro* culture. Therefore, it is not impossible that 100 % triploid plants would be obtained

from anther culture. The development of triploid pollen plants of Grenache might belong to this case. They could be derived from the multicellular mass developing from microspore (Fig. 2 e). Therefore they should be homozygous although this could not be confirmed because of the sterility of triploid plants.

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