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In ovulo embryo culture of stenospermocarpic grapes[1])

by

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Summary: Ovules of 14 seedless cultivars, collected 41—49 d after anthesis, were excised and *in vitro* cultured on 2 different media: NN + 1 μM GA_3 + 10 μM IAA and NN + 1 μM GA_3 + 20 μM IAA + 2 g/l activated charcoal. Plant development occurred at variable rates; the best percentages were found in Flame Seedless, Perlon, Imperatrice, Carina, Perlette and Ruby Seedless. Genotype resulted to be the main factor affecting ovule response to culture, but also the medium and the interaction genotype × medium influenced it. The addition of 2 g/l activated charcoal to the medium, tested on the cv. Perlette, and the type of added auxin (IBA, IAA, NAA), tested on the cvs Perlette and Sultanina, enhanced plantlet development.

Histological observations on flowers and berries of Perlette and Sultanina showed anomalies in embryo sac formation and also the presence of viable embryos in the ovules.

Key words: table grape, stenospermocarpy, tissue culture, embryo rescue, histology, breeding.

Introduction

In *Vitis vinifera* L., seedlessness is caused by stenospermocarpy or parthenocarpy (STOUT 1936). The stenospermocarpic grapes are favoured for their potentially larger berry size, than can be enhanced by girdling, thinning the cluster, or by spraying with gibberellins.

In these seedless grapes, pollination and fertilization occur but are followed by various degrees of embryo abortion (NITSCH *et al.* 1960; BARRITT 1970). Genetic control of stenospermocarpy is still under investigation (SPIEGEL-ROY *et al.* 1990).

The breeding of seedless grapes by traditional methods is hampered by the problem that seedless cultivars can be used only as male parent. *In vitro* culture of embryos or of fertilized ovules can prevent abortion and promote the embryo development. This technique allows breeders to cross seedless by seedless grapes, resulting in a higher frequency of seedless progeny; besides this can contribute to understand the genetic and physiological factors controlling stenospermocarpic seedlessness.

The first report concerning *in ovulo* embryo culture of stenospermocarpic grape was published in 1983 (CAIN *et al.*). Since then, the technique has been improved and successfully applied to intraspecific and interspecific crosses (SPIEGEL-ROY *et al.* 1985; GOLDY *et al.* 1988; BOUQUET and DAVIS 1989; TSOLOVA 1990; FERNANDEZ 1991). Results of the previous works pointed out that not only the composition of the culture medium but also the genotype affects the success of the culture.

The objectives of this investigation were: a) to optimize the technique for obtaining a high percentage of developing plantlets from ovule culture (avoiding the subsequent, labour intensive, excision of embryos from the ovules); b) to identify seedless cultivars from which ovules can be cultured more successfully; c) to investigate the effects of some components of the culture medium on ovule development.

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Materials and methods

a) Fruits obtained from open pollination of 14 cultivars were collected 41—49 d after anthesis. Their ovules were aseptically removed and cultured on the basal medium of NITSCH and NITSCH (1969) with added 1 μM GA_3 + 10 μM IAA (medium A, according to SPIEGEL-ROY *et al.* 1985) and NN + 1 μM GA_3 + 20 μM IAA + 2 g/l activated charcoal (medium B).

The tested cultivars were: Perlon, Flame Seedless, Pasiga, Nerona, Imperatrice, Carina, Argentina (from vineyards near Bari); Superior Seedless, Centennial Seedless, Giada, Dawn Seedless, Ruby Seedless (from vineyards near Rome); Perlette and Sultanina syn. Thompson Seedless (from vineyards near Turin).

b) The effects of activated charcoal (2 g/l) and of hormonal concentration (1 μM GA_3 + 10 μM IAA; 2 μM GA_3 + 20 μM IAA) compared to absence of charcoal and hormones were tested according to a factorial scheme using the same basal medium on ovules of Perlette collected 48 d after anthesis.

c) Ovules of Perlette and Sultanina were cultured 50 d after anthesis on the same basal medium with added 1 μM GA_3 and 10 μM of IAA or IBA or NAA.

In all the experiments berries were surface sterilized by immersion for 10 min in a 2.25 % chlorine solution containing surfactant, then rinsed in sterile water. Five ovules were placed in 90 mm × 15 mm Petri dishes, containing 20 ml medium. Petri dishes were sealed with Parafilm M and kept in a climatic chamber at 25 °C, with a photoperiod of 16 h and 50 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PPF. Three replications of 30 ovules were used for all the experiments. Observations on ovule development were carried out after 3 months of culture. Data were elaborated with ANOVA significance test.

After full growth of cotyledons and the formation of roots and sometimes of a few leaves, the plantlets were transferred to 30 mm × 200 mm test tubes containing 20 ml of MURASHIGE and SKOOG medium (1962) with salts at half strength, without hormones. When bearing 5—6 leaves they were planted in pots with a mixture of soil, peat and perlite, and acclimatized in a greenhouse.

Flowers of Sultanina and Perlette were collected at bloom; berries were collected 40—50 d after anthesis. This was done in order to observe the anomalies in embryo sac formation related to stenospermy and the stage of embryo development corresponding to the dates of *in vitro* culture. Samples were fixed in formalin-acetic acid-alcohol (FAA) and embedded in paraffin. Sections 10—12 μm thick were stained with Feulgen and light green and observed under a light microscope.

Results and discussion

Preliminary tests, performed on Perlette and Delight, indicated that ovules collected and cultured 41 and 52 d after anthesis give better results than ovules cultured at later or earlier dates, in accordance with the results reported by SPIEGEL-ROY *et al.* (1985) and TSOLOVA (1990).

The results of the experiment a) carried out on 14 cultivars pointed out (Tab. 1) that the ovules of some of them developed mainly callus (Pasiga, Perlon, Imperatrice, Carina, Perlette), while others (Argentina, Giada, Dawn S.) slightly enlarged and remained green but did not develop. Plantlet development occurred at variable rates, with the best percentages in Flame S., Perlon, Imperatrice, Carina, Perlette and Ruby S.

Genotype can be considered the main factor affecting the behaviour of cultured ovules, but also the medium and the interaction of genotype × medium can influence

Table 1

Type of ovule development (%) after 3 months of culture referred to genotype and medium; average of 3 replications, 30 ovules each. Culture medium: Nitsch and Nitsch (1969) added with 1 µM GA_3 and 10 µM IAA (medium A) or 1 µM GA_3, 20 µM IAA and 2 g/l activated charcoal (medium B). Sum of percentages of each combination can be > 100 because some ovules produced both callus and plantlet.

CULTIVAR	Medium A				Medium B			
	Darkened ovules	Green, enlarged ovules	Callused ovules	Ovules developing plantlet	Darkened ovules	Green, enlarged ovules	Callused ovules	Ovules developing plantlet
Pasiga	40.8	10.2	49	0	15.2	8.7	76.1	0
Flame	68.1	19.1	9.6	4.3	48	14	34	7
Perlon	44.6	2	51.5	6.9	26.6	0	69.1	11.7
Nerona	81.4	14.3	4.3	0	-1)	-	-	-
Argentina	21.3	55.3	23.4	0	29	43	26.9	1.1
Imperatrice	1	17.8	81.2	1	2.9	9.6	87.5	5.8
Carina	6.1	10.2	81.6	15.3	1	9.3	87.6	13.4
Perlette	18	0	80	8	19.2	0	77.8	4
Superior	29.5	3.4	67	1.1	52.2	6.7	41.1	0
Centennial	89	8.8	1.1	1.1	74.2	3.4	20.2	2.2
Dawn	57.6	23.2	19.2	0	40	27	33	0
Ruby	55	34	10	1	38.7	16.8	43.7	3.4
Giada	25	40.6	34.4	0	29.5	37.9	32.6	0
Sultanina	65.6	7.8	26.6	0	-1)	-	-	-

ANOVA SIGNIFICANCE TEST	genotype	medium	genotype x medium
darkened ovules	**2)	**	**
green, enlarged ovules	**	*	NS
callused ovules	**	**	**
ovules developing plantlet	**	NS	*

1) Not tested
2) **, *, NS: Significant for P=0.01, 0.05 and not significant, respectively.

it. Nevertheless the effect of the medium on embryo and plantlet development was not significant. In some cases both callusing and germination occurred, but observations clarified that embryos did not originate from callus.

Data from experiment b) (Tab. 2) revealed a significantly positive effect of the addition of 2 g/l activated charcoal to the medium on the percentage of developing plantlets, while the higher concentration of hormones as well as the interaction hormone level × presence of charcoal did not significantly affect the cultures. The presence of charcoal decreased the percentage of blackened, degenerated ovules, but did not reduce callusing (as noted by Cain *et al.* 1983 on a different cultivar with 1 g/l of charcoal).

Experiment c) demonstrated that the kind of auxin acts significantly on plant development, IAA being the most favourable and NAA giving the worst results (Tab. 3).

Table 2

Type of ovule development (%) for cv. Perlette after 3 months of culture referred to hormonal concentration and presence of charcoal; average of 3 replications, 30 ovules each. Sum of percentages of each formulation can be > 100 because some ovules produced both callus and plantlet.

	Darkened ovules	Green, enlarged ovules	Callused ovules	Ovules developing plantlet
No hormone no charcoal	58.1	0	41.9	6.4
1 µM GA_3 + 10 µM IAA no charcoal	26.4	0	73.6	4.2
2 µM GA_3 + 20 µM IAA no charcoal	27.3	1.1	70.5	1.1
no hormone 2 g/l charcoal	26.5	0	70.2	6.6
1 µM GA_3 + 10 µM IAA 2 g/l charcoal	17.8	0	77.1	10.1
2 µM GA_3 + 20 µM IAA 2 g/l charcoal	21.5	0	73.8	5.9

ANOVA significance test

	hormonal concentration	charcoal	hormones x charcoal
blackened ovules	NS	*	NS
green, enlarged ovules	NS	NS	NS
callused ovules	NS	NS	NS
germination	NS	*	NS

1) *, NS: Significant for P=0.05 and not significant, respectively.

Table 3

Percentage of developing plantlets from ovules after 3 months of culture with 3 types of auxin. Basal medium of NITSCH and NITSCH (1969) with added 1 µM GA_3 and 10 µM auxin. Averages of 3 replications, 30 ovules each.

Auxin	Perlette	Sultanina
IBA	3.3	0.8
IAA	4.2	1
NAA	0	0

ANOVA significance test

auxin type	significant for P = 0.05
genotype	not significant
auxin type x genotype	not significant

45 % of plantlets, developed from cultured ovules in all the experiments, could be transferred to soil. The hormone-free medium used in the second phase of the culture did not appear to be adequate and further investigations are needed.

Histological observations showed that at bloom about 50 % of ovules were anom-

alous for the absence of embryo sac or for the disorganized distribution of the nuclei in it. In addition, nucelli which protruded through the micropilar pole were frequently observed in Perlette.

At 40—45 d after anthesis about 20 % of the fertilized ovules showed the proembryo (Fig. a and b) whose diameter was 20—40 µm. It consisted of 6—20 cells, some of which appeared collapsed. In most cases it was surrounded by degenerating nucellus while the poorly developed endosperm was already degenerated.

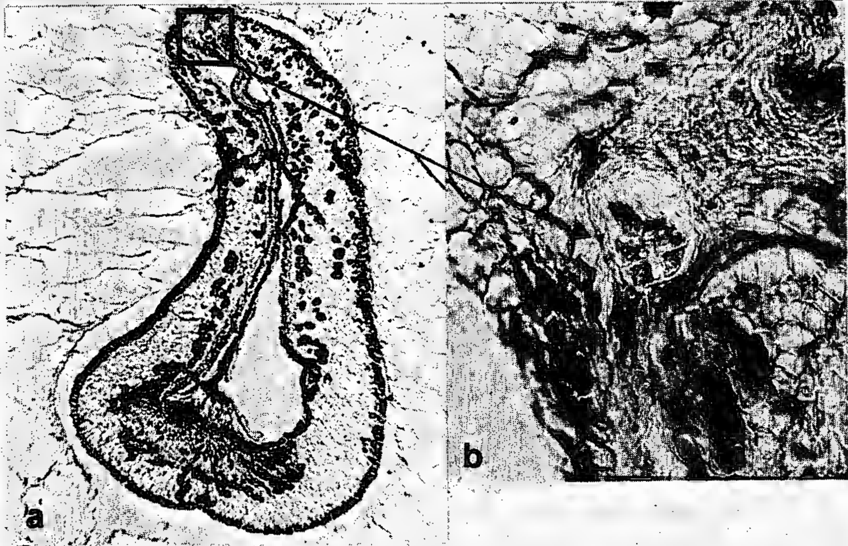

Figure: Degenerated ovule of cv. Perlette 45 d after anthesis (a: 40 ×) with pro-embryo surrounded by degenerating nucellus (b: 240 ×).

These findings confirm that, as stated by EMERSHAD *et al.* (1989), the embryos are arrested in development and remain viable even without the presence of a functional endosperm.

Our results confirm the possibility of obtaining plantlets from seedless grapes by means of ovule culture without embryo excision.

The genotype influence on the response to *in vitro* culture indicates the cultivars Flame S., Perlon, Imperatrice, Carina, Perlette and Ruby S. as having a greater propensity for *in ovulo* embryo culture.

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