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# *In-ovulo* embryo culture and seedling development of seeded and seedless grapes (*Vitis vinifera* L.)

by

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## Die Kultur von Samenanlagen zur Erzeugung von Embryonen und die Entwicklung von Sämlingen bei kernhaltigen und kernlosen Reben (*Vitis vinifera* L.)

Zusammenfassung. — Aus normalerweise abortierenden Samenanlagen kernloser Rebsorten konnten normale Embryonen und Keimpflanzen gewonnen werden. Die Samenanlagen kernhaltiger und kernloser Sorten wurden isoliert und auf 10 verschiedenen Medien kultiviert, wobei der Entnahmezeitpunkt vom Tag der Anthese bis auf 101 d nach dem Aufblühen — insgesamt 12 verschiedene Termine — ausgedehnt wurde. Samenanlagen, die bis zu 14 d nach der Anthese isoliert worden waren, zeigten ein normales Wachstum. 24 und 31 d nach der Anthese isoliert, entwickelten sich die Samenanlagen zu äußerlich normalen Samen, die aber nur degenerierte Embryonen und Endospermreste enthielten. Lebensfähige Embryonen wurden gefunden, wenn die Embryonen frühestens 38 d nach der Anthese in Kultur genommen worden waren. Die Samenanlagen einer kernlosen Sorte, die 52 d nach dem Aufblühen isoliert worden waren, keimten und erzeugten gesunde Sämlinge. Bei Selbstbestäubung bildeten 7 von 13 kernlosen Klonen in den kultivierten Samenanlagen vitale Embryonen. Aus den kultivierten Samenanlagen frei abgeblühter oder untereinander gekreuzter kernloser Klone entstanden ebenfalls lebensfähige Embryonen und Sämlinge. Die Ergebnisse weisen darauf hin, daß viele stenospermokarp kernlose Reben lebensfähige Embryonen hervorbringen können, wenn sich die Samenanlagen nur in einem angemessenen Milieu entwickeln können. Die Konsequenzen für die Züchtung kernloser Rebsorten werden diskutiert.

## Introduction

Seedless table and raisin grapes are preferred by consumers in much of the world. However, there are very few commercially acceptable seedless cultivars. The breeding of stenospermocarpic[2]) seedless cultivars is hampered by several problems: 1) Seedless cultivars can be used only as male parents; 2) the frequency of seedlessness in progenies of crosses between seeded × seedless is very low, averaging about 15 % (7); 3) seeds of normal-seeded early-maturing cultivars often germinate very poorly; 4) there is an insufficient understanding of the genetic and environmental factors controlling seedlessness; 5) the 3-year or longer generation time and large land area needed make it difficult to grow large populations and conduct genetic studies.

Seedlessness appears to be controlled by a single or a few recessive genes (7, 17, 19). However, the frequency of seedlessness in progenies is very variable and does not conform to normal genetic ratios (7, 13).

The occasional presence of seeds or viable embryos in normally seedless phenotypes (11, 12) suggests that it is reasonable to assume that embryos of seedless grapes are genetically sound. Their abortion may result from a physiological imbalance in the

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[2]) In this paper only the stenospermocarpic form of seedlessness will be considered.

parental tissues during a critical stage of ontogeny. Several reports indicate that differences in auxin (3) and gibberellin (2, 8, 10) concentrations exist between fruits of seeded and seedless grapes. These differences may be involved in the expression of seedlessness.

Embryo culture has long been used to overcome embryo abortion in stone fruits (18). Ovule culture has also been used to overcome embryo and endosperm abortion of intergeneric cotton hybrids (4, 15, 16), intergeneric cereal grain hybrids (6), and haploids from cereal grains (5). The development of an *in vitro* technique to culture normally abortive ovules from seedless grapes would permit hybridization among seedless cultivars which can now be accomplished only indirectly over two generations. If seedlessness is a recessive trait (7), such crosses should result in a much higher frequency of seedlessness in the progenies.

This report describes the *in vitro* culture and subsequent germination of normal and abortive grape ovules.

## Materials and methods

In the first set of experiments, the seeded cultivars selected were Cardinal, which has poor seed germination because the fruit ripens before the embryos are mature and Red Malaga, which has a high germination rate. The seedless cultivars used were C35-33, which has a large abortive ovule, and Sultanina (Thompson Seedless), which has a very small abortive ovule.

Bloom occurred on May 15, 1979, for Cardinal, C35-33 and Red Malaga and on May 18, 1979, for Sultanina. Samples were taken at anthesis, 3, 6, 9, 14 and 17 d postanthesis, and then at weekly intervals until 52 d after anthesis. A final sample was taken at 101 d after anthesis when fruit of all cultivars was past veraison. Sampling of Sultanina was discontinued 17 d after anthesis because we originally thought that ovule abortion had occurred by this time.

Berries were surface sterilized by immersing for 20 min in 2.6 % sodium hypochlorite containing 0.1 % Tween-20 surfactant. Ovules were removed under aseptic conditions and placed on 10 media combinations. For the first 6 sampling dates, ovules were placed in 2 petri dishes of each medium with 3 ovules of each cultivar per dish. Beginning 24 d after anthesis, ovules were placed individually into test tubes using 5 tubes per medium per cultivar. The media used were Murashige and Skoog (MS) (9), a high salt medium; half-strength (macrosalts) Murashige and Skoog (1/2 MS); Smith's

Table 1

Number of germinating seeds derived from 50 ovules cultured at 52 and 101 d after anthesis

Anzahl gekeimter Kerne aus jeweils 50 kultivierten Samenanlagen, die 52 und 101 d nach dem Aufblühen isoliert worden waren

| Cultivar | d after anthesis | |
|---|---|---|
| | 52 | 101 |
| Cardinal | 2 | 16 |
| C35-33 | 13 | 19 |
| Red Malaga | —[1] | 34 |

[1] Lost to contamination.

medium (14), a low salt medium developed for stone fruit embryo culture; White's medium (20), a low salt rooting medium and Stewart and Hsu's medium (15), developed for cotton ovule culture. Each medium was used with and without 0.1 % activated charcoal added to give 10 medium combinations. All media contained 30 g sucrose/l and 7 g agar/l. All cultures were grown at a temperature of approximately 25 °C and illuminated for 18 h/d at 800 μW/cm².

Table 2

Numbers of viable embryos obtained from cultured ovules of self pollinated seedless grape clones

Anzahl lebensfähiger Embryonen aus kultivierten Samenanlagen selbstbestäubter Rebenklone

| Cultivar | Number of viable embryos |
|---|---|
| Thompson Seedless | 0 |
| Black Monukka | 0 |
| P59-183 | 0 |
| B31-164 | 0 |
| C58-22 | 0 |
| P100-111 | 0 |
| P60-58 | 1 |
| A24-124 | 2 |
| B1A-27 | 2 |
| Flame Seedless | 2 |
| C78-68 | 7 |
| B4-86 | 7 |
| Arkansas 1105 | 53 |

40 ovules per cultivar were cultured except for Arkansas 1105 where 116 open pollinated ovules were cultured.

Table 3

Percent of viable embryos produced by cultured ovules of a seedless grape crossed to seedless and seeded pollen parents

Prozentsatz lebensfähiger Embryonen, die aus den kultivierten Samenanlagen einer kernlosen Sorte nach Kreuzung mit kernhaltigen und kernlosen Pollenspendern hervorgingen

| Cross | Number of ovules cultured | Percent of viable embryos[1]) |
|---|---|---|
| Seedless × seeded | | |
| C35-33 × Petite Sirah | 277 | 25.3 a |
| Seedless × seedless | | |
| C35-33 Selfed | 182 | 20.9 a |
| C35-33 × Perlette | 377 | 10.1 b |
| C35-33 × Flame Seedless | 310 | 5.5 c |

[1]) Means followed by the same letter are not significantly different at $\chi^2$ 105. $\chi^2_1$, .05.

All cultured ovules were stratified in the dark at 4 °C from September 29, 1979 until December 20, 1979 after which they were returned to the laboratory for germination. Germination was considered complete on February 21, 1980. The remaining ungerminated ovules were weighed and then dissected and examined for the presence of embryos and embryo-like structures. Excised embryos were placed into vials containing 2 ml of liquid MS medium supplemented with 2 ppm benzyladenine (BA) (1). Those embryos in which the cotyledons expanded and became green were considered viable.

In 1980, 13 seedless grapes were self pollinated (Table 2), and several seedless × seeded and seedless × seedless hybridizations were made (Table 3). The berries were harvested on July 29, 1980. All ovules were excised and placed on White's medium with 4 % sucrose. The ovules were allowed to develop at approximately 25 °C for 2 weeks then they were stratified in the dark at 4 °C until mid December when they were returned to the laboratory. On February 3, 1981, the ovules were dissected and examined for the presence of embryos.

## Results and discussion

Ovules of both seeded and seedless cultivars excised at 0—6 d after anthesis grew very slowly and abnormally on all media. All ovules became round and eventually produced prolific callus with those on the high salt MS medium producing the most callus. Ovules on MS an ½ MS media survived longer than those on other media. The addition of 0.1 % activated charcoal reduced tissue browning, reduced callusing, and reduced media discoloration. Most ovules placed upon the media containing activated charcoal remained green throughout the experiment. Dissection of the ovules revealed that all were hollow and contained no visible embryo or endosperm remnants even when the outer integument tissues remained green. The callus was produced by the outer layers of the outer integument.

At 9 and 14 d after anthesis, some ovules of all cultivars except Sultanina enlarged, elongated, assumed the shape of a normal seed, but failed to enlarge to full size. Again, those on media without activated charcoal callused excessively and eventually died while those on media containing 0.1 % activated charcoal remained green and produced less callus. Ovules on MS and ½ MS media produced more callus than those on the other media. None of the ovules germinated and dissection revealed that all were hollow.

Beginning 24 d after anthesis, some ovules of all 3 cultivars developed into what appeared to be normal, mature seeds. Again no germination occurred and dissection revealed no embryo or endosperm in any ovules. Ovules cultured 31 d after anthesis contained only degenerating embryo and endosperm remnants. One viable embryo was found in the C35-33 ovules cultured 38 d after anthesis; Cardinal and Red Malaga contained only degenerating remnants. Beginning at 45 d after anthesis Cardinal, C35-33 and Red Malaga had 6, 4 and 5 viable embryos, respectively.

When cultured at 52 d postanthesis, both Cardinal and C35-33 produced normal-appearing seeds which germinated (Table 1). All ovules of Red Malaga sampled at 52 d postanthesis were lost to contamination. C35-33 ovules cultured at 52 d postanthesis developed into seeds weighing 30.8 mg which is similar in size to mature seeds from Red Malaga (32.5 mg). They were much larger than typical abortive seeds from mature C35-33 fruit (10.3 mg) and the seedlings from cultured ovules were healthy and vigorous.

50 fully mature seed each of Cardinal, C35-33 and Red Malaga cultured at 101 d after anthesis produced 16, 19, and 34 seedlings, respectively. Germination of C35-33

ovules cultured at 101 d after anthesis was greater than those cultured at 52 d postanthesis even though weight of ovules cultured at 52 d was over three times greater than normal C35-33 ovules or ovules cultured at 101 d post anthesis. The increased weight of C35-33 ovules cultured at 52 d was due mainly to an increase in the amount of endosperm present. By 101 d after anthesis the ovules had become sclerified and no enlargement occurred. Medium composition appeared to have no effect on ovule development or germination for ovules cultured beyond 24 d after anthesis. It was interesting to note that only those ovules placed on the agar surface germinated, while those embedded in the medium failed to germinate even though they contained viable embryos. The reason for this effect is not known.

To compare the germination of cultured and uncultured C35-33 ovules, 4,000 seeds of C35-33 were extracted from mature fruit, stratified, and planted in sand using standard procedures for germinating grape seeds in the breeding programm. 40 seedlings were obtained from the 4,000 seeds giving a germination percentage of only 1.0 %. The resulting seedlings were very weak and many subsequently died. By comparison, C35-33 ovules cultured at 52 d had greater than 25 % germination and produced healthy, vigorous seedlings.

6 of the 12 clones of seedless grapes produced viable embryos from cultured self pollinated ovules (Table 2). Also nearly half of the open-pollinated Arkansas 1105 ovules produced viable embryos. This indicates that embryos of seedless grapes are genetically sound and are capable of developing normally if they are placed in the proper environment.

Crosses between seedless grapes also produced viable embryos (Table 3). The different pollen parents produced significantly different numbers of viable embryos when crossed to C35-33. Both the seeded Petite Sirah and the large abortive seeds of C35-33, produced more embryos than Perlette and Flame Seedless which have very small abortive seeds.

The frequency of seedlessness in progenies of seeded × seedless crosses varies with the seedless pollen parent used (7). Our data indicate that the seedless pollen parent also influences the frequency of viable embryos in crosses between seedless grapes. The genetic makeup of the zygote and/or the endosperm therefore, has at least some influence on embryo survival and development in addition to the effects of maternal tissues and environmental conditions.

The production of viable embryos and plants from 6 different self pollinated seedless grapes and the production of viable embryos from crosses between different seedless grapes indicate that viable embryos can be produced by many stenospermocarpically seedless grapes if the developing ovule is placed in the proper environment.

The use of this technique will allow production of hybrid progenies having as both parents, seedless genotypes which should facilitate genetic studies leading to a clearer understanding of the inheritance and nature of the seedless fruit. The culture of seedless ovules will also permit direct recombination of grape germplasm which was heretofore impossible and it will hopefully result in a higher frequency of seedlessness in seedling populations thus increasing the efficiency of breeding programs.

## Summary

Normal embryos and seedling plants were obtained from normally abortive ovules of seedless grapes. Ovules of seeded and seedless cultivars were excised and cultured on 10 media at 12 dates between anthesis and 101 d postanthesis. Ovules cultured from anthesis until 14 d postanthesis grew abnormally. At 24 and 31 d after anthesis, ovules

developed into normal-appearing seeds, but they contained no noticeable embryo or endosperm. Viable embryos were found in excised ovules cultured beyond 38 d after anthesis. Ovules of 1 seedless grape cultured 52 d postanthesis germinated and produced healthy seedlings. Self pollinated ovules from 7 to 13 seedless clones produced viable embryos when cultured. Open pollinated ovules and crosses between seedless clones also produced viable embryos and seedling plants. This indicates that stenospermocarpic seedless grapes can produce viable embryos. Implications for breeding of seedless grapes are discussed.

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