Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon

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**ABSTRACT**

Somatic embryogenesis and subsequent diploid plants have been obtained from anthers of *Vitis vinifera* Cabernet-Sauvignon, a cultivar so far considered as recalcitrant to *in vitro* regeneration. Anther enclosing microspores near the first pollen mitosis were found to be the most responsive. However, from a practical point of view anther length proved to be an easier criterion for determining the optimal physiological anther stage. Calli derived from the same anther somatic tissues produced embryoids only when cultured on a medium supplemented with casein hydrolysate. Glutamine and adenine were found to stimulate this embryoid production. Evidence is presented that early removal of cotyledons increases the frequency of normal development of embryoids into plantlets.

**Abbreviations:**
- MS: Murashige and Skoog medium (1962)
- 2,4-D: 2,4-dichlorophenoxyacetic acid
- NAA: 1-naphtaleneacetic acid
- BA: 6-benzylaminopurine

**INTRODUCTION**

Some commercially important grapevine cultivars (*Vitis vinifera*) present cultural defects leading to economic losses. *In vitro* techniques appear to be the only tool available for the genetic improvement of these varieties grown in vineyards where hybridization processes are not allowed. Regeneration of plantlets has been obtained by cultivation of nucellus in *Vitis vinifera* (MULLINS and SRINIVASAN 1976, SRINIVASAN and MULLINS 1980) and by cultivation of vegetative tissues essentially in interspecific hybrid grapevines (FAVRE 1977, KRUL and WORLEY 1977, HIRABAYASHI 1985). Embryoid and plantlet production from cultured anthers of *Vitis* long reported to be unsuccessful (MULLINS 1971, GRESSHOFF and DOY 1974) has been achieved more recently (RAJASEKARAN and MULLINS 1979, BOUQUET et al. 1982, HIRABAYASHI 1982). However, the cultivar Cabernet-Sauvignon, used all over the world for the production of high quality red wines, appears to be recalcitrant to anther culture.

Various physiological factors have been found to enhance the embryoid formation of numerous plants. For *in vitro* androgenesis through anther culture, the developmental stage of the anther seems to be important (SUNDERLAND et al. 1977, SIBI et al. 1979, DUNKELL 1986). Additionally, the presence in the culture medium of reduced nitrogen such as ammonium ion and certain amino acids was found to stimulate *in vitro* embryogenesis of carrot in particular (HALPERIN and WETHERELL 1965, WETHERELL and DOUGALL 1976, KAMADA and HARADA 1979).

The purpose of this paper is therefore to examine the influence of cultural conditions, such as anther length and the addition to the culture medium of various nitrogen compounds, in order to increase callus and embryoid yield of Cabernet-Sauvignon.

**MATERIALS and METHODS**

The methods for anther culture and induction of somatic embryos were adapted from BOUQUET et al. (1982). Cool-stored (4°C) cuttings of 3 clones (n° 15, 1565 and 337) of Cabernet-Sauvignon, provided by Mr Pouget (INRA Bordeaux) were grown in greenhouse according to the method of MULLINS (1966). Inflorescences were collected and chilled for 72 hours at 4°C. The flowers were then surface-sterilized (10 min) with a filtered 7 % (w/v) solution of calcium hypochlorite containing Tween 20 (0.1 % v/v) and rinsed several times with sterile distilled water.

Callus initiation. The anthers, 0.2 to 1 mm long, were excised from the flowers and cultured during 28 days in 3 cm Petri dishes containing half-strength MS (1962) medium solidified with 0.7 % agar and supplemented with sucrose (2 % w/v), 2,4-D (4.5 μM) and BA (1 μM).

Callus proliferation. The anthers were transferred to medium supplemented with NAA (0.5 μM), BA (1 μM) and casein hydrolysate (250 mg/l) and cultured during one month.

The various nitrogen compounds examined were added before autoclaving to these two media.

Embryogenetic callus proliferation. Calli were subcultured each month on a regeneration medium lacking casein hydrolysate and supplemented with NAA (0.5 μM) and BA (1 μM).

Embryogenic callus proliferation. Calli were individually transferred to test tubes containing BA (0.4 μM) in order to develop plantlets.

All the cultures were maintained at 24°C under a 16 h illumination regime (100 μE m⁻² s⁻¹), except the callus initiation performed in the dark. The percentage of anthers producing callus was determined at the end of the first subculture upon embryogenetic medium.

The percentage of calli producing embryoids was determined after three subcultures upon embryogenetic medium.
RESULTS and DISCUSSION

1) EFFECT OF ANTHER LENGTH

For three clones of Cabernet-Sauvignon (n° 15, n° 1565, n° 337), the effect of anther length on callus and embryoid yield is presented in Table I.

Table I: Effect of anther length on callus and embryoid initiation of three clones (n° 15, 1565, 337) of Vitis vinifera cv. Cabernet-Sauvignon.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Anther length (mm)</th>
<th>Number of anthers cultured</th>
<th>% Anthers producing callus</th>
<th>%a in embryogenic calli</th>
<th>%a in embryoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.3</td>
<td>850</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.3 to 0.5</td>
<td>750</td>
<td>8.7</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.5 to 0.7</td>
<td>450</td>
<td>11.6**</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>300</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0.3</td>
<td>600</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.3 to 0.5</td>
<td>1400</td>
<td>25**</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>0.5 to 0.7</td>
<td>1550</td>
<td>19.2**</td>
<td>1.7</td>
<td>76.2</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>1200</td>
<td>6.8</td>
<td>1.2</td>
<td>3.7</td>
</tr>
<tr>
<td>337</td>
<td>0.3</td>
<td>360</td>
<td>25</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.3 to 0.5</td>
<td>820</td>
<td>25.8**</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.5 to 0.7</td>
<td>980</td>
<td>43.8**</td>
<td>1.8</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>1300</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- %a: calculated compared to the number of anthers producing callus.
- Significantly different from the other classes (2x2 contingency test)
- * at 0.05 level of probability
- ** at 0.01 level of probability

For each clone the most suitable developmental stage of anther for producing callus was remarkably and significantly constant and corresponded to the two intermediate classes: 0.3 to 0.5 mm and 0.5 to 0.7 mm. These anther classes initiating most calli were also the most efficient at producing embryoids.

A comparison of the three clones pointed out differences in their capacities to initiate calli and embryoids. Clone n° 1565 was found to be significantly (P = 0.05) the least effective especially in producing calli. These calli had low embryogenic capacities (figure 1). The two other clones n° 15 and n° 337 were more efficient in both callus and embryoid production. However, the percentages of embryogenic calli were in general rather weak since they never went beyond 1.8. An occasional high embryoid yield was observed (76.2 %) displaying the high embryogenic capacity of certain calli (figure 2).

These data confirm those of BOUQUET et al. (1982), showing that the ability of anthers to produce calli and embryoids depends on genotype.

Until now the most suitable anthers retained for grapevine somatic embryogenesis have been identified by their microspore state (RAJASEKARAN and MULLINS 1979, HIRABAYASHI 1982). According to the present results it seems that anther length is the best practical parameter. By the way, it was found that callus was produced by the anther wall and/or the connective and not by the microspores (RAJASEKARAN and MULLINS 1983).

Thus, in the following experiments all the cultured anthers of Cabernet-Sauvignon were 0.5 mm long. This size was easily identified by a color shift of the anther wall, from green to yellow.

2) EFFECT OF CASEIN HYDROLYSATE, AMINO ACIDS AND ADENINE ON CALLUS AND EMBRYOID INITIATION

Anthers of clone n° 337 have been used in an experiment designed to define, primarily, the effect of casein hydrolysate added to the proliferation medium on callus and embryoid yield.

The effect of casein hydrolysate on callus initiation was not appreciable; with or without casein hydrolysate the percentages of calli were similar. However, the aspect of calli was greatly different, and without casein hydrolysate none of the calli, uniformly brown, were able to induce embryoids (figures 3 and 4).