Somatic embryogenesis of *Vitis vinifera* L. (cv. Sugraone) from stigma and style culture

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Summary

Somatic embryo and plant regeneration were induced from stigma and style culture of grapevine (*Vitis vinifera* L. cv. Sugraone). To obtain somatic embryogenesis, explants were cultured on Nitsch and Nitsch basal medium (NN) supplemented with 88 mM sucrose and various combinations of the auxin β-naphthoxyacetic acid (NOA, 0-10 µM) and the cytokinin 6-benzylaminopurine (BA, 0-9 µM). Growth regulators (BA and NOA) in the culture medium were essential for induction of somatic embryogenesis since explants incubated on hormone-free medium never regenerated somatic embryos. Usually, the regenerated somatic embryos become visible as small white globular structures on the surface of the callus 3-4 months after culture initiation. The best embryogenic response (27 %) was obtained when stigma and style explants were cultured on NN medium supplemented with 0.5 µM NOA and 9 µM BA. Somatic embryos developed into plantlets when transferred to a hormone-free semisolid NN medium. 35 % of primary somatic embryos showed secondary embryogenesis.

**Key words**: Anthers, grapevine, plant regeneration, proembryogenic masses, *Vitis vinifera*.

**Abbreviations**: BA: 6-benzylaminopurine; NN: Nitsch and Nitsch medium; NOA: β-naphthoxyacetic acid; PEMs: proembryogenic masses.

Introduction

Genetic improvement of grapevine by conventional breeding is impeded by long juvenile periods and inbreeding depression (Jayasankar et al. 2000). Therefore alternative approaches involving the use of somatic embryogenesis would be of great benefit for genetic improvement programs and virus elimination (Goussard et al. 1991, Goussard and Wiid 1992, Bouquet and Torregrosa 2003).

Reliable protocols for the regeneration of *Vitis vinifera* L. somatic embryos have been reported in literature (Martinelli and Grivaudo 2001). Somatic embryos have been regenerated from different explant types such as unfertilized ovules, anthers, immature zygotic embryos, immature leaves, tendrils, immature ovaries, leaf discs and filaments (Mullins and Srinivasan 1976, Mauro et al. 1986, Stamp and Meredith 1988 a, b, Salunkhe et al. 1997, Nakano et al. 2000, Das et al. 2002, Nakajima and Matsuda 2003). Although several types of tissues have been successfully used, anthers are considered the most suitable explant for the regeneration of somatic embryos; therefore, they are currently used to induce the regeneration of somatic embryos in many genotypes (Bouquet and Torregrosa 2003). The number of genotypes from which somatic embryogenesis can be induced is increasing but still limited (Nakajima and Matsuda 2003). Therefore new efficient protocols involving the use of alternative explants for the induction of somatic embryogenesis offer a highly attractive system for physiological and genetic studies.

In the present work, a typical explant (anthers) used in previous work for the regeneration of *V. vinifera* somatic embryos, and alternatively floral tissues (stigmas and styles) were compared in order to improve the regeneration of plants through somatic embryogenesis.

Material and Methods

**Plant material**: Cultures were initiated from anthers and stigma-style explants. Immature flowers from field-grown plants of *V. vinifera* cv. Sugraone were used. Flowers length: 1.5 - 2.5 mm were collected when the petals were still closed (about 1 week before flowering). They were surface-sterilised with 75 % ethanol-water for 3 min and 0.6 % sodium hypochlorite and 0.05 % Tween 20 (Sigma) for 15 min. Flowers were then rinsed three times in sterile distilled water for 5 min. Each corolla was removed under sterile conditions using a stereo microscope. Anthers and stigma-style explants were excised with a sterile forceps and sharp blade from the flowers and placed in the petri dishes containing the different media. Stigma-style explants were placed vertically onto plastic Petri dishes with the cut surface in contact with the medium. Five explants were placed in each Petri dish and 50 explants were used per treatment.

**Media and culture conditions**: In all the experiments the culture medium consisted of Nitsch and Nitsch (1969) (NN) basal salts and vitamins with the addition of 88 mM sucrose. Three groups of experiments were carried out to compare the embryogenic potential of anther and stigma-style explants. The first set of experiments was carried out to compare culture media reported in literature...
for the regeneration of somatic embryos in 4 species of the genus *Vitis* (Gray 1992, Martinelli et al. 1993, Tsolova and Atanassov 1994, Harst 1995). The 4 different hormonal combinations to test the embryogenic potential of stigma-style explants are presented in Tab. 1. Explants incubated without growth regulators were used as control. In a second set of experiments 16 different growth regulator combinations were used: the auxin β-naphthoxyacetic acid (NOA, Sigma N-3019, 0, 1, 5 and 10 µM) associated with the cytokinin 6-benzylaminopurine (BA, Sigma B-3274, 0, 0.5, 4.5 and 9.0 µM). In a third set of experiments stigma and style explants were collected during the third year and incubated only on 9 µM BA + 5 µM NOA or 9 µM BA + 10 µM NOA supplemented media.

After the addition of growth regulators the pH of the medium was adjusted to 5.6 before autoclaving at 121 °C for 20 min. All the media were gelled with 0.8% (w/v) agar. Plastic Petri dishes, 55 mm in diameter and 15 mm high were used for explant culture, each containing 8 ml of medium. Five explants were placed in each Petri dish and 10 Petri dishes were used per treatment (50 explants). Petri dishes were sealed with laboratory sealing film. Explants and calli were subcultured at 60-d-intervals and maintained in a climatic chamber at 25 °C, 16 h·d⁻¹ light (photosynthetic photon flux density: 40 µmol m⁻² s⁻¹ cool-white fluorescent lamps).

**Plant regeneration and in vivo acclimatization:** Explants and calli that differentiated somatic embryos were transferred to hormone-free NN medium supplemented with 88 mM sucrose (NN-) and incubated for 1-2 months to allow embryo proliferation and development. To allow germination of the embryos regenerated from anther and stigma-style explants, 10 embryos were placed on 8 ml of NN-medium in a plastic Petri dish (55 x 15 mm). Two hundred embryos per type of explant were used to calculate the percentage of embryo germination and conversion of embryos into plants. Embryos were considered as germinated when there was root extension and hypocotyl elongation. After germination, to allow the conversion of embryos into plants, two germinated somatic embryos were transferred to NN-medium (each 100 x 20 mm Petri dish containing 25 ml of medium). Embryos were considered converted into plants when the shoot apex developed and formed new expanded leaves. Plantlets were transferred to autoclaved Jiffy® peat pellets and maintained in a basal heating bench at 25 °C and at high relative humidity (95-98%). To estimate the growth of regenerated plants the average number of nodes and plant length was scored. In this experiment 40 plants for each explant type were used. The mean number of nodes and plant length were calculated after two months of growth in the basal heating bench. Subsequently, the plants were pricked into pots containing sterile soil, transferred to the greenhouse and exposed to natural daylight conditions at 22/27 °C (night/day).

**Results**

The 4 different hormonal combinations (reported in literature and chosen to test the embryogenic potential of stigma-style explants, Tab. 1) promoted the formation of soft translucent creamy-white callus. In fact, about 1-2 weeks after culture initiation in all tested media we observed callus proliferation, while the hormone-free medium did not promote callus formation as expected. Callus became visible at the basal part of the style and consisted of cell clusters or single cells of variable size and shape.

About 3-4 months after culture initiation white somatic embryos were differentiated at the surface of callus regenerated from responsive explants (Fig. 1). Tab. 2 shows the embryogenic response of explants incubated on 4 different media scored after 6 months of incubation. Both explants regenerated somatic embryos only in the medium supplemented with 0.9 µM BA and 5 µM NOA. The attempts to induce somatic embryogenesis from anther and stigma-style explants cultured on the control (hormone-free medium) and the other media reported in literature were not successful (Tab. 2).

The second set of experiments was carried out to compare different levels of growth regulators which in the first set of experiments gave the best results. As in the first set of experiments, within the culture media used for the regeneration of somatic embryos in 4 species of the genus *Vitis*, only

<table>
<thead>
<tr>
<th><strong>Species</strong></th>
<th><strong>Explant type</strong></th>
<th><strong>Auxin compound and concentration</strong></th>
<th><strong>Cytokinin compound and concentration</strong></th>
<th><strong>GA₃</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. rotundifolia</em></td>
<td>Immature zygotic embryos</td>
<td>0.9 µM BA</td>
<td>5.0 µM NOA</td>
<td></td>
<td>Gray 1992</td>
</tr>
<tr>
<td><em>V. rupestris</em></td>
<td>Leaf and petiole</td>
<td>4.4 µM BA</td>
<td>0.5 µM 2,4-D</td>
<td></td>
<td>Martinelli et al. 1993</td>
</tr>
<tr>
<td><em>V. vinifera</em></td>
<td>Stenospermocarpic ovules</td>
<td>1 µM KIN</td>
<td>10 µM IAA</td>
<td>1 µM GA₃</td>
<td>Tsolova and Atanassov 1994</td>
</tr>
<tr>
<td><em>V. thunbergii</em></td>
<td>Leaf discs</td>
<td>4 µM TDZ</td>
<td>20 µM NOA</td>
<td></td>
<td>Harst 1995</td>
</tr>
</tbody>
</table>

* BA = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; GA₃ = gibberellic acid; IAA = 3-indoleacetic acid; KIN = kinetin; NOA = 2-naphthoxyacetic acid; TDZ = Thidiazuron.*
the hormonal combination 0.9 \( \mu M \) BA and 5 \( \mu M \) NOA gave best results, 16 different combinations of these two growth regulators were used. Tab. 3 shows the embryogenic response of anther and stigma-style explants incubated on these media. The embryogenic response depended on explant type and growth regulator combination. Both explants regenerated somatic embryos, although they showed a different embryogenic aptitude. Stigma-style explants showed highest embryogenic potential as compared to anthers. The embryogenic response of explants was also affected by the hormonal composition of the medium. The higher percentage of stigma-style explants producing somatic embryos were 20 \% on 9.0 \( \mu M \) BA + 5 \( \mu M \) NOA supplemented medium and 16 \% on 9.0 \( \mu M \) BA + 10 \( \mu M \) NOA. The percentage of embryogenic anthers was 8 \% on 4.5 \( \mu M \) BA + 5 \( \mu M \) NOA supplemented medium and 5 \% on 4.5 \( \mu M \) BA + 10 \( \mu M \) NOA (Tab. 3). Addition of BA and NOA was necessary to induce regeneration of somatic embryogenesis, in fact it was never observed in explants incubated on hormone-free medium (Tab. 3).

A high percentage (about 60 \%) of embryos showed a bipolar organisation, after incubation on growth regulator-free medium, they usually passed through globular, heart, torpedo and early cotyledon stages, and finally germinated embryos (Fig. 2). During embryo development we observed a change in colour from white to green.

The average number of primary somatic embryos produced directly from the callus derived from anther and stigma-style explants was low, i.e. 9 and 6, respectively. However, repetitive somatic embryogenesis - which comprises successive cycles of somatic embryogenesis induced from the hypocotyl cells of regenerated plants - allowed the rescue of many embryos. About 35 \% of primary somatic embryos produced secondary somatic embryos. Secondary somatic

<table>
<thead>
<tr>
<th>Hormonal combination</th>
<th>Auxin concentration</th>
<th>Cytokinin concentration</th>
<th>GA3</th>
<th>Embryogenic anthers (%)</th>
<th>Embryogenic stigmas-styles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9 ( \mu M ) BA</td>
<td>5.0 ( \mu M ) NOA</td>
<td></td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4.4 ( \mu M ) BA</td>
<td>0.5 ( \mu M ) 2,4-D</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1 ( \mu M ) KIN</td>
<td>10 ( \mu M ) IAA</td>
<td>1 ( \mu M ) GA3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4 ( \mu M ) TDZ</td>
<td>20 ( \mu M ) NOA</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Control hormone free medium</td>
<td></td>
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</table>
embryos were regenerated from the area surrounding the hypocotyl (Fig. 3). Embryogenic callus consisted of white proembryogenic masses (PEMs). When PEMs were maintained on induction medium the cytokinin and the auxin in the medium had an inhibitory effect on their development into somatic embryos. We observed that embryogenic callus can be subcultured for long time (> 2 years) maintaining its embryogenic potential.

Discussion

The present study indicates a high embryogenic potential of stigma and style tissues of cv. Sugraone. Although regeneration of somatic embryos from anther culture in grapevine is generally considered efficient and is the most commonly used method (BOUQUET and TORREGROSA 2003), stigma and style explants showed highest embryogenic potential as compared to anthers. In both explants the tested growth regulators were required for induction of somatic embryogenesis. However, the cytokinin and auxin levels required for the induction of somatic embryogenesis, depended on the explant type. Under our experimental conditions, anthers required lower levels of cytokinin (4.5 \( \mu M \)) in the culture medium as compared with stigmas and styles (9 \( \mu M \)).

The highest embryogenic response was observed in stigma and style explants cultured on NN medium supplemented with 5 \( \mu M \) NOA and 9 \( \mu M \) BA (20 and 27 % of responsive explants).

Another part of the flower, the ovary, has already been reported as a valuable explant for induction of somatic embryogenesis in different species of \textit{Vitis} (GRAY and MORTENSEN 1987, NAKANO \textit{et al.} 1997) and in some cases the embryo-
genic potential of this explant was higher as compared to anther explants (MARTINELLI et al. 2001a, 2003).

Since embryogenic calli obtained from stigma and style culture may have been derived solely from cells of somatic origin, the regenerated embryos have a genetic constitution that is identical to that of the plant source, excluding somaclonal variation. Usually, anther cultures can produce embryos that arise from diploid cells of the connective tissue and are therefore of somatic origin with a genome identical to that of the mother plant (FAURE et al. 1996, SALUNKHE et al. 1999). However, it has been reported that the embryos regenerated from anther culture may arise from the haploid cells of gametic origin (BOUQUET and TORREGROSA 2003).

Secondary embryogenesis from primary somatic embryos is a crucial aspect for a long time preservation of embryogenic cultures (VILAPLANA and MULLINS 1989, MARTINELLI et al. 2001 b). Under our experimental conditions a high percentage (35 %) of primary somatic embryos regenerated secondary somatic embryos. Secondary embryogenesis has several advantages when compared to primary somatic embryogenesis, such as a high multiplication rate, independence of explant availability (i.e. flowers or immature leaves are available only for a short period), moreover embryogenic cell lines can be maintained for several years (RAEMAKERS et al. 1995).

We have observed that the callus formed from stigma and style culture is either embryogenic or not. It is usually easy to distinguish between non-embryogenic and embryogenic callus on the base of its colour and morphology. Embryogenic callus consists of white PEMS.

In the present work, phenotypically normal cv. Sugraone plants have been regenerated. However, the embryogenic potential of stigma-style explants of other genotypes should be tested. Moreover, genetic identity of regenerated plants should also be confirmed in the field and at molecular level. Genetic identity of regenerated plants has been tested. Moreover, genetic identity of regenerated plants should also be confirmed in the field and at molecular level according to the mother plant (FAURE et al. 1996, SALUNKHE et al. 1999). However, it has been reported that the embryos regenerated from anther culture may arise from the haploid cells of gametic origin (BOUQUET and TORREGROSA 2003).

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References


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