Large-scale production of somatic embryos as a source of hypocotyl explants for Vitis vinifera micrografting

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Summary

To the standard methods currently used to make grapevine virus-free, apex micrografting on hypocotyls of somatic embryos is proposed as an alternative procedure. The study defines optimal conditions to produce hypocotyl fragments suitable for micrografting. Interruption of the process by storage of tissues or embryos at low temperature (+ 4 °C) was assessed at different stages and for durations up to 6 months. Best procedure to produce somatic embryos were: long-term maintenance of embryogenic cultures on C1 medium (5 μM 2,4-D + 1 μM BAP, solidified with 4 g·l⁻¹ agar and 4 g·l⁻¹ Phytagel); differentiation of embryogenic callus for 2 months on C2 medium (5 μM NOA + 1 μM BAP, gelling agents same as above); transfer of single embryos on plant growth regulator-free medium for 2-3 weeks for germination. At different steps of the process, embryogenic tissues or differentiated embryos can be stored for up to 180 d for some cultivars. Micrografting assays were performed with various types of embryo and with apices from several V. vinifera cultivars. White to slightly coloured hypocotyls, excised from embryos germinated in darkness, gave best results for micrografting, while hypocotyl shape had little influence. For all genotypes tested the success rate ranged from 18 to 30 %.

Key words: grapevine, virus sanitation, micrografting, somatic embryos, embryogenesis.

Introduction

For grapevine, in vitro heat therapy was the first method to eliminate virus (Galzy 1963). Thermotherapy is efficient against fanleaf virus (GFLV) but the eradication of more heat-resistant viruses like grapevine leafroll or fleck virus-infected plants, but this method proved highly genotype-dependent and its effectiveness varied according to the source of embryos and virus type.

An alternative method is in the micrografting of a shoot tip or an axillary bud apex (200-500 μm) onto internode cuttings (Ayuso and Pesta-Iglesias 1976) or a hypocotyl fragment from a seedling (Bass et al. 1976, Engelbrecht and Schwertfeger 1979). Using the latter method, routine sanitation procedures were developed for cloning selection programs by Benin and Grenan (1984) and Cupidi and Barba (1986), but the grafting success was very low mainly due to the browning of tissues extracted from seedlings. Various improvements, i.e. in vitro seed germination (Cupidi and Barba 1988), provisional conservation of buds (Cupidi and Barba 1993) or apex pre-treatment with plant growth regulators (Ben Abdallah et al. 1996) were suggested but the generalisation of the technique remains difficult. In this study, we tried to find out the best conditions to produce grapevine somatic embryos providing hypocotyl fragments highly compatible for micrografting.

Material and Methods

Plant material: Embryogenic cultures of 4 Vitis vinifera cultivars (Ugni blanc, Cot, Marselan and Portan) were induced and maintained on C1P medium according to Torregrosa (1998). To recover isolated somatic embryos the method described by Torregrosa (1998) was used with slight modifications. Freshly (30 d) subcultured calluses were transferred after fractioning onto C2P medium. After 60 d single embryos (2-4 mm, torpedo-heart stage) were carefully excised and plated horizontally onto Internode medium (5 μM BAP, solidified with 4 g·l⁻¹ agar and 4 g·l⁻¹ Phytagel) and 4 g·l⁻¹ Phytagel) and 4 g·l⁻¹ Phytagel) ; differentiation of embryogenic callus for 2 months on C2 medium (5 μM NOA + 1 μM BAP, gelling agents same as above) ; transfer of single embryos on plant growth regulator-free medium for 2-3 weeks for germination. At different steps of the process, embryogenic tissues or differentiated embryos can be stored for up to 180 d for some cultivars. Micrografting assays were performed with various types of embryo and with apices from several V. vinifera cultivars. White to slightly coloured hypocotyls, excised from embryos germinated in darkness, gave best results for micrografting, while hypocotyl shape had little influence. For all genotypes tested the success rate ranged from 18 to 30 %.

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combination of both (4 g l⁻¹ Phytagel® + 4 g l⁻¹ agar) were evaluated with C1 medium (TORREGROSA 1998). To encourage the formation of pro-embryos and the differentiation, C2 (C medium, 5 µM NOA + 1 µM BAP) combined with 0, 2.5, 5 or 7.5 µM ABA or C4 (C medium, 5 µM IAA + 1 µM BAP) were compared. For embryo germination and development, MS/2 based medium (MURASHIGE and SKOOG 1962) combined with 0 or 2.5 µM BAP was used. To evaluate the effect of light on embryo development, isolated embryos (torpedo and higher stages, length: 2-4 mm) were first incubated for 15 d in darkness on plant growth regulator-free MS/2 medium and then exposed for 7 d to various light intensities (0, 15, 30 or 45 µmol·s⁻¹·m⁻², photoperiod: 14 h). To test the effect of stopping the process of embryo production, embryogenic callus, differentiated single embryos or germinated embryos were stored at (+4 °C) for 15-180 d. Unless otherwise specified, cultures were incubated in darkness at 26 ± 1°C.

Experiments followed a completely randomized factorial design with 10 replicates per cultivar x combined-media treatment with one replicate being represented by a 55 mm plate containing 10 callus fragments (approximately 50 mg fresh weight each). During the different stages of embryo development and during the subcultures, the number of replicates was maintained at the same level: 10 plates x 10 callus or embryos, each experiments being run until germination. In this study, more than 62 000 embryos were brought to the stage of germination.

The following variables were determined: number of germinated embryos, i.e. number of embryos without necrosis but with growing cotyledons, cylindrical hypocotyls and developing roots; fresh weight (single embryos) and dry weight (pooling the embryos of each plate); hypocotyl aspect: length, shape (straight, slightly curved or twisted), vitrified or not; colour (white, green, red, pink); cotyledon number and shape (normal, abnormal); rhizogenesis: number and total length of main roots.

Micrografting experiments: Terminal and axillary buds were collected from infected and healthy grapevines growing in a climatic chamber at 32 °C for more than 2 months. After sterilization (10 % calcium hypochlorite, 15 min; 3 washes with sterilized water, 10 min) buds were carefully dissected under binocular to excise shoot apices (200-500 µm). Apices were immediately grafted onto rooted somatic embryos and grown on MS/2 medium without growth regulator, in culture tubes (length: 150 mm). The V. vinifera cultivars Syrah cl. 174 (infected with grapevine rupestris stem pitting virus), Syrah cl. 300 (healthy); Gamay cl. 656 (GRSPaV-infected); Prunelard cl. E2 (GFLV-infected) and Prunelard cl. E3 (GFLV- and GfKV-infected). The effect of several factors on micrografting was investigated: colour and shape of somatic embryos used to extract hypocotyl explants, position of the graft at the hypocotyls (head section/side): 60-100 micrografts were analysed for each genotype x treatment combination.

Results and Discussion

Effect of the genotype: Among the 4 cultivars tested, Portan showed best capacities to produce and maintain embryogenic cultures. The callus produced with this genotype showed typical embryogenic characters (Fig. 1 b): white to light yellow, friable but not pulverulent nor

Fig. 1: a) Development of cvs Cot and Portan embryo clusters after 15 d in culture on C0, C2, C2 + ABA and C4 media (bar: 10 mm); b) Embryogenic callus of cv. Portan after 5 weeks in culture on C1P medium (bar: 5 mm); c) Germinated somatic embryo of cv. Portan ready to use for micrografting (bar: 10 mm); d) 3009 cl. 111 apex grafted on a cv. Portan hypocotyl, 30 d in culture (bar: 10 mm).
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Effect of the genotype on somatic embryo development

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Germination</th>
<th>Dry matter</th>
<th>Hypocotyls</th>
<th>Cotyledons</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>CI*</td>
<td>%</td>
<td>CI</td>
<td>%</td>
</tr>
<tr>
<td>Marselan</td>
<td>52</td>
<td>5.6</td>
<td>8.5</td>
<td>0.7</td>
<td>18.0</td>
</tr>
<tr>
<td>Ugni Blanc</td>
<td>95</td>
<td>1.9</td>
<td>7.3</td>
<td>0.4</td>
<td>17.0</td>
</tr>
<tr>
<td>Cot</td>
<td>100</td>
<td>-</td>
<td>23.2</td>
<td>7.2</td>
<td>24.0</td>
</tr>
<tr>
<td>Portan</td>
<td>99</td>
<td>0.6</td>
<td>5.7</td>
<td>0.4</td>
<td>48.8</td>
</tr>
</tbody>
</table>

* Confidence interval at p = 0.05; ** Standard error.
case low temperatures positively affected the development of embryogenic callus or developing embryos (Tab. 3, Fig. 2). These results agree with those of Bouquet et al. (1986) and Krul and Worley (1977). Moreover, there is experimental evidence that, although not beneficial to embryo development, exposure to 4 °C can be used to store embryogenic cultures or embryos after germination. For all cultivars, embryogenic callus can be preserved up to 30 d without loss of germination capacity (Fig. 2). Up to 30 d, the response of embryogenic callus varied with regard to the cultivar, Cot showing an extreme susceptibility while Portan embryos maintain high germination ability up to 6 months of cold storage. Furthermore cold storage of single differentiated embryos was possible up to 30 d without dramatic reduction of the germination capacity (Tab. 3).

M i c r o g r a f t i n g a s s a y s : Experiments on micrograft position showed that in addition to be much more convenient, side grafting (Fig. 1d) was more successful (19 % versus 14.2 % with Syrah cl. 300) than head section grafting. The colour of the hypocotyls were of major importance to obtain high survival rates of graftings, best results being obtained with white or little coloured hypocotyls extracted from embryos germinated in darkness (Fig. 3). Furthermore, stems and leaves from the grafted apex developed considerably faster (1-2 months) with hypocotyls excised from white embryos than from coloured ones (2-5 months). Consequently, embryos germinated in darkness represent the best source of explants for micrografting. Hypocotyl shape and the utilisation of apices extracted from virus-infected plants (Syrah cl. 174, Prunelard E2 and E3) did not strongly influence the percentage of grafting.

The best procedure to produce somatic embryo hypocotyls is presented in Fig. 4. According to the genotype, the rate of successful micrografting using somatic embryo explants could routinely reach 18-30 %, a high percentage compared to that obtained with seedling hypocotyls (in general less than 10 %, unpubl.). Large-scale experiments are currently performed with apices from plants infected with main grapevine viruses or combinations of these viruses in order to precise the conditions of this method.

T a b l e 2
Effects of light intensity (µmol·m⁻²·s⁻¹; 7d) on embryo development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Light int.</th>
<th>Germination % CI*</th>
<th>Hypocotyls Length cm SE**</th>
<th>Cotyledons Normal % CI</th>
<th>White % CI</th>
<th>Total length cm SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cot</td>
<td>0</td>
<td>75 3.5 33.5 5.1 1.5 0.1 100.0 - - -</td>
<td>45 8.4 2.4 1.3 0.1 27.0 5.5 0.0 -</td>
<td>0 100 - 60.0 4.1 2.2 0.1 29.0 5.3 26.0 3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Portan</td>
<td>0</td>
<td>100 - 66.0 4.1 2.2 0.1 29.0 5.3 26.0 3.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Confidence interval at p = 0.05; ** Standard error.

T a b l e 3
Effect of cold storage of isolated embryos on embryo development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Days at 4 °C</th>
<th>Germination % CI*</th>
<th>Hypocotyls Not twisted % CI</th>
<th>Length cm SE**</th>
<th>Cotyledons Normal % CI</th>
<th>Roots No./emb. n SE</th>
<th>Total length cm SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cot</td>
<td>0</td>
<td>99 0.6 55.3 20.3 1.6 0.7 11.1 1.9 2.8 1.2 4.6 2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Portan</td>
<td>0</td>
<td>100 - 70.0 21.1 2.0 0.6 10.0 1.8 2.1 0.9 6.1 2.8</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

* Confidence interval at p = 0.05; ** Standard error.
Acknowledgements

Authors would like to thank A LAIN B OUQUET and P HILIPPE C HATELET for critically reading the manuscript.

References


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Received January 5, 2004