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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 %.

K e y w o r d s : 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 rugose wood complex requires other methods. Meristem culture on a medium containing growth regulators was proposed by Goussard (1981) as an efficient method to produce virus-free grapevines. Buciumeanu and Visoiu (2000) and Bottalico *et al.* (2000, 2003) improved this method by altering the balance of growth regulators and the number of subcultures. To increase efficiency, Hatzinikolakis *et al.* (1993) and Gribaudo *et al.* (1997), combined meristem culture with *in vitro* thermotherapy. Several authors (Goussard *et al.* 1990, Schaefers *et al.* 1994, Gribaudo *et al.* 2003, Popescu *et al.* 2003) obtained healthy plants through somatic embryogenesis regeneration of grapevine fanleaf, 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 Peña-Iglesias 1976) or a hypocotyl fragment from a seedling (Bass *et al.* 1976, Engelbrecht and Schwerdtfeger 1979). Using the latter method, routine sanitation procedures were developed for clonal 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

P l a n t m a t e r i a l : 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 MS/2 medium without plant growth regulators (diameter of plates: 100 mm). To stimulate germination of embryos, plates were incubated for 15 d in darkness and then, to obtain greening cotyledons, exposed to light for 7 d (15 µmol·m^{-2}·s^{-1}, photoperiod: 14 h).

P r o d u c t i o n o f s o m a t i c e m b r y o s : Several combinations of culture conditions and media were tested with the 4 cultivars listed above. For embryogenic callus maintenance, the effect of 4 different solidification formulations, *i.e.* Sigma Phytagel (5 or 8 g·l^{-1}), agar-agar (8 g·l^{-1}) or a

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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 storaged 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

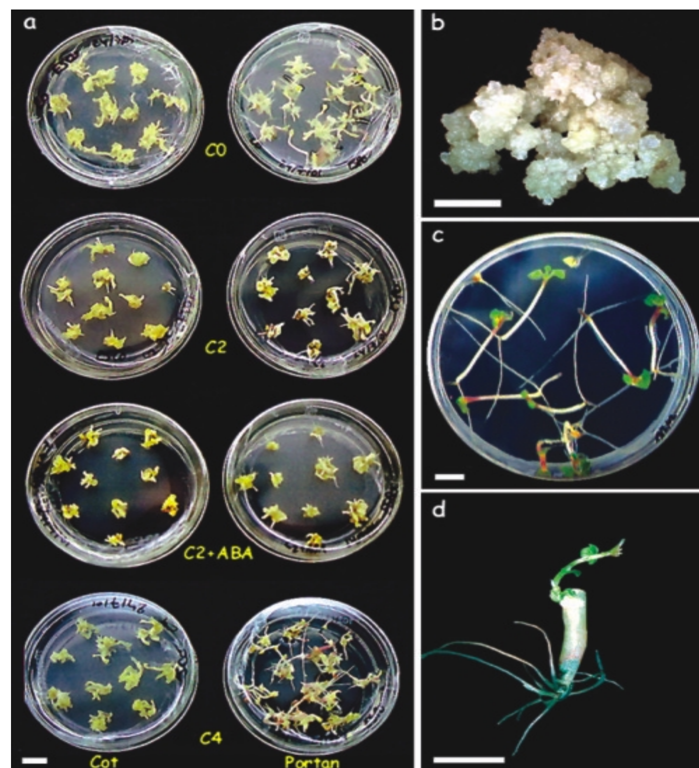

Fig. 1: **a**) Development of cvs Cot and Portan embryo clusters after 15 d in culture on C0, C2, C2 + ABA and C4 media (bar: 10 mm); **b**) Embryogenic callus of cv. Portan after 5 weeks in culture on C1P medium (bar: 5 mm); **c**) Germinated somatic embryo of cv. Portan ready to use for micrografting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

hyperhydric, composed of opaque small embryonic masses without epiderm. With this genotype, the rate of multiplication could be maintained at high level (x 10 / 4-6 weeks) for more than 5 years. The callus of Marselan had the same structure, but growth was slower. The callus of Cot was very similar in structure to those of Portan, but being a grey-brown it yielded little transferable tissue for subculturing. Ugni Blanc produced the highest rate of differentiated callus; being somewhat friable it made long-term maintenance of embryogenic cultures very difficult.

During germination, a strong genotypic effect was observed (Tab. 1). Marselan showed a very low ability to produce germinating embryos which, in addition, were very short, twisted and with abnormal cotyledons. Ugni Blanc and Cot showed very high germination rates but, within developed embryos, the percentage of straight and long embryos was rather low. Portan was the best cultivar in terms of germination and number of straight and long hypocotyls.

Effect of the solidification formulation: The nature of the gelling agent has previously been reported to be a major factor for development of *in vitro* tissues of Quercus (Pierik *et al*. 1997). Among the 4 solidification formulation assessed in this study, A4P4 was the best in term of callus growth and quality. The use of Phytagel® only led to good-looking callus with a low hydricity level and necrosis, but with a significantly lower growth rate than with A4P4, especially with P8. With A8, callus showed highest growth rates but a very poor ability to develop embryonic structures when transferred to step B, probably because of their hyperhydricity. The type and concentration of the gelling agent appeared to be also very important. The use of Phytagel® combined with agar was the best treatment to maintain embryogenic callus growth without hampering the regeneration of embryos, confirming results of Perl *et al*. 1995.

Effect of plant growth regulators: Growth regulators are reported to encourage grapevine embryo development: BAP in promoting caulinar meristem organization (Gray and Mortensen 1987) or shoot axillary proliferation of somatic embryos (Torregrosa 1994), ABA in preventing precocious germination (Goebel-Tourand *et al*. 1993, Perl *et al*. 1995, Faure *et al*. 1996) and IAA in stimulating embryo differentiation and rhizogenesis (Perl *et al*. 1995, Faure *et al*. 1998). In this study, we compared different formulations of C2 medium (addition of 0, 2.5, 5 or 7.5 µM ABA) with the C4 medium during callus cultures and the MS/2 medium (addition of 0 or 2.5 µM BAP) during single embryo development.

The clusters of differentiated embryos extracted from C2 medium developed better when cultured on C0 or C4 medium (Fig. 1a). Moreover, embryos grown on C4 medium were easy to isolate from clusters, thus increasing the number of embryos transferable to the germination step (data not shown). With single embryos extracted from C2 medium, the addition of 2.5 µM BAP to MS/2 medium dramatically reduced rhizogenesis and decreased the number of exploitable embryos (data not shown). In summary any of the conditions tested was better than the standard procedure in supporting differentiation of embryogenic calluses or the germination of embryos.

Effects of light: The effect of light on grapevine somatic embryo development is only scarcely documented. When applied during the first days of germination, light was previously reported to induce negative effects on the texture and shape of somatic embryos of perennial plants (Baker *et al*. 1994). In grapevine, most authors use light at the latest stage of germination when shoot development is needed to convert embryos to rooted plantlets. In this study, the effect of 7 d of light exposure to 0, 15, 30 or 45 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (photoperiod: 14 h) on the development of single embryos (torpedo and upper stages, length: 2-4 mm) was evaluated. Results showed that grapevine embryo morphology is noticeably affected by light exposure. For all cultivars, light decreased length and proportion of straight hypocotyls and increased the coloration (pink to dark red) of embryos (Tab. 2), indicating that light exposure should be avoided when hypocotyls are to be extracted for micrografting.

Effect of the cold storage: The use of cold storage was proposed for several perennial species to overcome low germination rate (Merkle 1995). In grapevine, the effect of low temperature on germination of *V. vinifera* somatic embryos was assessed by Trautmann and Burger (1996) who found that short expositions to low temperature (5 d) can improve significantly the germination rate for Sultanina but not for two other cultivars, indicating a genotype-dependent response.

The effect of low temperatures was evaluated after its application at 3 phases of somatic embryo development: (i) embryogenic callus, (ii) single ungerminated embryos and (iii) rooted embryos ready to use for micrografting. In no

Table 1

Effect of the genotype on somatic embryo development

	Germination		Dry matter		Hypocotyls				Cotyledons		Roots			
					Not twisted		Length		Normal		No./emb.		Total length	
Genotype	%	CI*	%	CI	%	CI	cm	SE**	%	CI	n	SE	cm	SE
Marselan	52	5.6	8.5	0.7	18.0	3.4	0.3	0.3	16.3	6.6	1.7	1.0	2.9	1.7
Ugni Blanc	95	1.9	7.3	0.4	17.0	2.0	1.3	0.6	11.0	3.8	3.0	1.8	4.2	2.4
Cot	100	-	23.2	7.2	24.0	2.5	0.9	0.6	3.0	0.9	2.7	1.8	1.0	0.7
Portan	99	0.6	5.7	0.4	48.8	2.5	2.0	0.8	35.3	2.0	2.2	1.6	5.3	3.0

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

Table 2

Effects of light intensity (µmol·m^{-2}·s^{-1}; 7d) on embryo development

Genotype	Light int.	Germination %	Germination CI*	Hypocotyls Not twisted %	Hypocotyls Not twisted CI	Hypocotyls Length cm	Hypocotyls Length SE**	Hypocotyls White %	Hypocotyls White CI	Cotyledons Normal %	Cotyledons Normal CI
Cot	0	75	3.5	33.5	5.1	1.5	0.1	100.0	-	-	-
	15	75	3.4	31.7	2.7	1.3	0.1	29.1	3.4	1.2	0.8
	30	79	2.5	34.5	2.4	1.3	0.1	41.8	4.4	4.8	1.6
	45	58	4.5	11.5	2.6	1.1	0.1	27.0	5.5	0.0	-
Portan	0	100	-	61.0	4.1	2.5	0.1	92.0	1.6	21.0	1.5
	15	100	-	50.0	4.5	1.9	0.2	38.0	2.6	18.0	2.7
	30	100	-	66.0	4.1	2.2	0.1	29.0	5.3	26.0	3.9
	45	100	-	35.0	4.0	1.7	0.1	23.0	3.5	15.0	2.5

* 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).

Micrografting assays: 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.

Table 3

Effect of cold storage of isolated embryos on embryo development

Genotype	Days at 4 °C	Germination %	Germination CI*	Hypocotyls Not twisted %	Hypocotyls Not twisted CI	Hypocotyls Length cm	Hypocotyls Length SE**	Cotyledons Normal %	Cotyledons Normal CI	Roots No./emb. n	Roots No./emb. SE	Roots Total length cm	Roots Total length SE
Cot	0	99	0.6	55.3	20.3	1.6	0.7	11.1	1.9	2.8	1.2	4.6	2.5
	15	96	1.4	24.8	26.7	1.6	0.4	3.2	1.4	2.6	1.5	2.6	2.0
	30	90	3.3	50.0	27.3	1.6	0.5	8.1	2.6	2.8	1.6	3.3	2.2
	45	4	1.4	83.3	28.9	1.8	0.4	0.0	-	4.0	0.8	2.2	1.3
	60	25	3.7	45.0	36.1	1.7	0.5	4.2	2.3	3.7	1.6	3.3	1.8
Portan	0	100	-	70.0	21.1	2.0	0.6	10.0	1.8	2.1	0.9	6.1	2.8
	15	100	-	68.0	22.0	2.0	0.8	16.0	2.3	2.0	1.0	5.5	2.8
	30	98	1.2	77.3	12.2	1.7	0.7	18.3	1.5	1.9	0.9	4.6	2.6
	45	63	5.8	80.0	21.6	1.6	0.7	8.3	3.2	2.0	1.0	5.4	2.4
	60	0	-	-	-	-	-	-	-	-	-	-	-

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

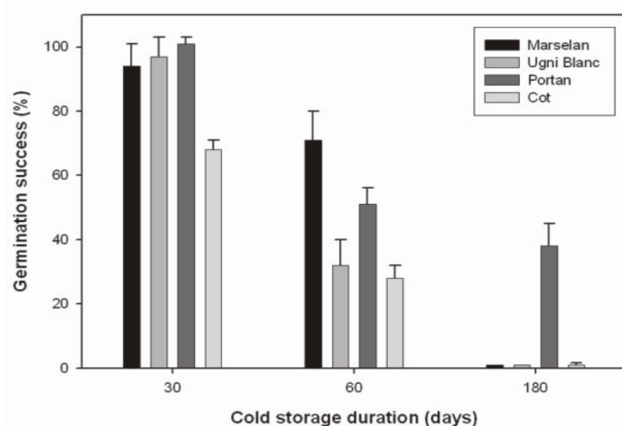

Fig. 2: Effect of cold storage of embryogenic callus, on germination of somatic embryos (no storage = 100 %, means of 10 replicates ± SE).

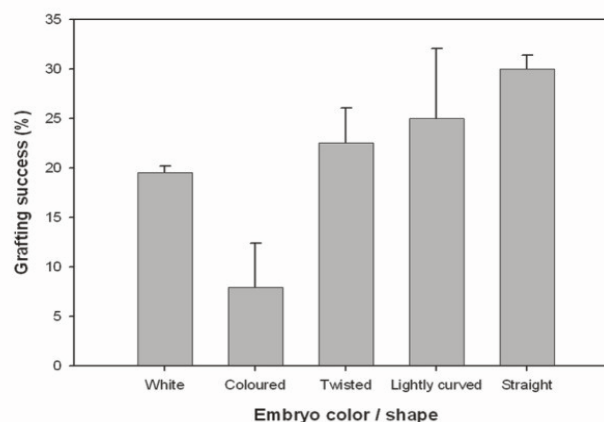

Fig. 3: Effect of hypocotyl colour and shape on the rate of grafting cvs Syrah cl. 174, Syrah cl. 300, Prunelard cl. E2 and E3 apices (60-100 explants/experiment/genotype).

Step	Medium	Explant type	Duration	Cold storage
Maintenance Multiplication	C1(A4P4)	Embryogenic callus	30-45 d	
Differentiation	C2(A8)	Whole calluses	60 d	30 d → 180 d (Portan)
Post-diff.*	C4(A8)	Embryo clusters	7 d	
Germination	MS/2(A8)	Isolated embryos	14-21 d	30 d.
Hypocotyl extraction		Germinated embryos		

* Depending on the cultivar.

Fig. 4: Process of somatic embryo regeneration to produce hypocotyls for micrografting.

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