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Efficient plant regeneration through somatic embryogenesis from anthers and ovaries of six autochthonous grapevine cultivars from Galicia (Spain)

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ABSTRACT

An efficient protocol for plant regeneration through somatic embryogenesis was developed for the first time in five autochthonous grapevine cultivars (Treixadura, Torrontés, Mencía, Merenzao and Brancellao) from Galicia (north-western Spain). Improvements of the induction protocol for the cv. Albariño in respect to previously reported data were also made. Media containing NN salts and MS vitamins supplemented with combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) were effective in inducing somatic embryogenesis. The addition of casein hydrolysate produced the best results for up to four cultivars (Albariño, Treixadura, Merenzao and Brancellao). Somatic embryogenesis was also induced in explants collected during the binucleate pollen microsporogenesis stage (R3 flower stage) of all cultivars with the exception of Treixadura, suggesting that under appropriate conditions explants can display longer windows of competence. Transfer of embryogenic callus to differentiation medium produced callus proliferation and somatic embryo development proliferation by secondary embryogenesis. However, an extensive process of precocious embryo germination was observed reducing the efficiency of secondary embryo proliferation. This situation was overcame by the use of differentiation medium lacking growth regulators (DM1 medium), which allowed reducing precocious germination by half on average and improving embryo proliferation by secondary embryogenesis. Transfer of normally developed, ungerminated isolated embryos to germination medium allowed obtaining very high percentages of embryo germination (up to 97% in Mencía, more than 87% averaging all cultivars). A comparison of plant conversion between precociously and normally germinated embryos showed that precocious germination in differentiation medium reduced plant conversion, even at high rates depending on the cultivar (from 93% to 39% in Brancellao, from 86% to 61% averaging all cultivars).

1. Introduction

Somatic embryogenesis in the grapevine was initially developed many years ago (Martinelli and Gribaudo, 2001). The main applications include the isolation of natural somatic mutants that arise during grapevine vegetative propagation (Boss and Thomas, 2002; Franks et al., 2002), improvement through exploitation of somaclonal variation (Kuksova et al., 1997; Desperrier et al., 2003), germplasm cryopreservation (Gray and Compton, 1993) and viral disease eradication (Goussard et al., 1991; Goussard and Wiid, 1992). In addition to utilizing somatic embryos in genetic transformation for plant improvement, large scale mutant production from somatic embryos through insertional mutagenesis has became an interesting goal for genomic programs devoted to assigning gene function, relying on the recent availability of drafts of the genome sequence of the grapevine (Jaillon et al., 2007; Velasco et al., 2007).

Somatic embryos can be obtained from various explants, mainly reproductive organs like ovaries (Kikkert et al., 2005; López-Pérez et al., 2005), stigmas (Morgana et al., 2004), anthers (Gribaudo et al., 2004; Kikkert et al., 2005), and whole flowers (Gambino et al., 2007). Although less common, somatic embryos have also been obtained from tissues derived from vegetative structures, like leaves and petioles (Martinelli et al., 1993; Das et al., 2002), tendrils (Salunkhe et al., 1997) or stem nodal explants (Maillot et al., 2006).

Although the number of *Vitis* species and cultivars from which somatic embryogenesis protocols are available is continuously

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, benzyladenine; NOA, naphthoxyacetic acid; IAA, indoleacetic acid; GA3, gibberellic acid; DAPI, 4′,6-diamidino-2-phenylindole; CIM, callus induction medium; DM, differentiation medium.

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increasing (Martinelli and Gribaudo, 2001), it is not yet a routine technology, in particular for the most valuable genotypes (Gribaudo et al., 2004). The main bottlenecks include large experimental variations due to the genotype and differential responses due to the interaction of several physiological factors involving the selected explant, its developmental stage, and the chemical factors included in the culture medium. Additional limitations of this technology include difficulties in producing mature, correctly developed somatic embryos that are amenable to normal plant conversion at high rates from embryogenic callus (Coutos-Thevenot et al., 1992; Perrin et al., 2001).

Galicia is a Spanish region with a long viticultural tradition that produces quality wines based on a rich autochthonous grapevine germplasm with several well-known cultivars and other local cultivars of limited production but great potential. The application of somatic embryogenesis to this germplasm is practically nil, regardless of very recent efforts with the cultivar Albariño (González-Benito et al., 2009; Vidal et al., 2009), which is probably the most famous quality white wine-producing cultivar from this region. In this paper, we report the development of complete protocols for plant regeneration through somatic embryogenesis for six important cultivars from Galicia (three of white berries and three of black berries), including some improvements for the induction frequency in the cultivar Albariño. Our work was focused on determining the appropriate developmental stage of the explants for somatic embryogenesis induction, as well as in determining the best conditions for somatic embryo differentiation and proliferation in order to reduce the phenomenon of somatic embryo precocious germination. A comparison of the potential for plant conversion of somatic embryos precociously germinating in differentiation medium with their counterparts germinating normally in germination medium was also made.

2. Materials and methods

2.1. Plant material

Adult field-grown plants of *Vitis vinifera* L. cvs. Albariño, Treixadura, Torrontés, Mencía, Brancellao and Merenzao were selected for this study in the grapevine collection at the Centro de Formación y Experimentación de Viticultura y Enología de Ribadumia (Galicia, north-western Spain). Inflorescences at stage H on the Baggiolini phenological scale corresponding to separated clusters were collected over a three-week period during April–May 2007. Flower developmental stages (R1, R2, and R3) were also determined by the microsporogenesis stage observed microscopically after anther squashing in the presence of DAPI with the aid of a stereomicroscope (Leica MZ10F). Stages R1 and R2 correspond respectively to stages V and VI as defined by Gribaudo et al. (2004), but the R3 stage corresponds to a later binucleate microspore stage (Fig. 1). Collected flower clusters were washed twice with distilled water containing a drop of detergent for 5 min, chilled at 4 °C for 4–6 days and then sterilized as described by Kikkert et al. (2005) prior to immature anther and ovary dissection to be used as explants for induction of somatic embryogenesis.

2.2. Callus induction and maintenance

Callus induction medium (CIM) consisted of NN (Nitsch and Nitsch, 1969) salts and MS (Murashige and Skoog, 1962) vitamins. Combinations of the growth regulators 2,4-D and BA along with two levels of sucrose and pH (Table 1) were tested for the initiation of embryogenic callus from anthers and ovaries of all six grapevine cultivars selected. The pH of the medium was adjusted with NaOH prior to autoclaving at 98 kPa and 121 °C and all media were solidified using 0.3% gelrite (Duchefa Biochemie, Haarlem, Netherlands). The effect of casein hydrolysate (0.1%) was also tested using CIM-1 (Table 1). Anthers and ovaries were placed on 90 mm diameter polystyrene Petri plates containing 25 mL of CIM. Cultures were maintained at 23 ± 1 °C in continuous darkness and subcultured onto fresh medium every 30 days. After embryogenic callus formation, cultures were routinely maintained on the original induction medium.

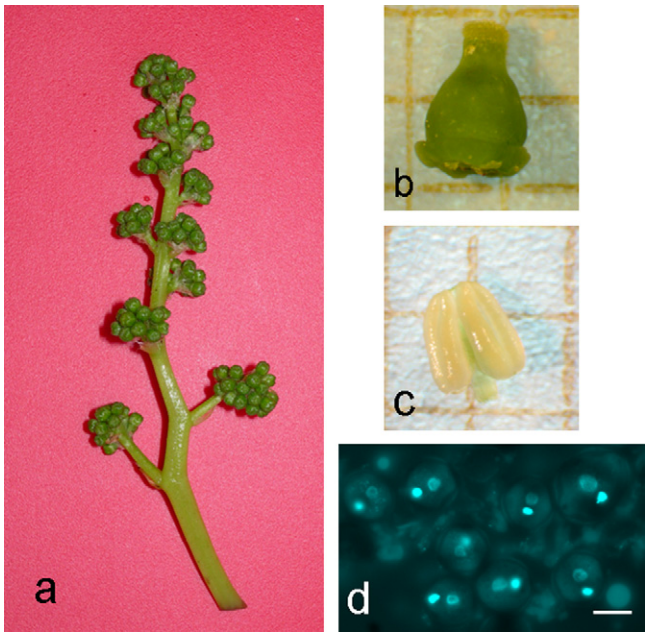

Fig. 1. (a) Inflorescence, (b) ovary explant, (c) anther explant and (d) DAPI-stained binucleate microspores at the R3 developmental stage in the grapevine cv. Mencía. White bar represents 10 μm.

2.3. Embryo differentiation

Embryogenic calli with or without visible embryos were subcultured on 90 mm diameter polystyrene Petri plates containing 25 mL of embryo differentiation medium (DM) consisting of CIM basal medium supplemented with 10 μM naphthoxyacetic acid (NOA), 1 μM BA, 20 μM indoleacetic acid (IAA), 0.6% sucrose and 0.25% activated charcoal. Medium without growth regulators (DM1) was also tested. The pH of the medium was adjusted to 5.8 with NaOH prior to autoclaving at 98 kPa and 121 °C and the medium was solidified using 1% gelrite (Duchefa). The cultures were maintained under the same conditions described for callus induction.

2.4. Embryo germination and plant regeneration

For germination, white embryos were cultured for 30 days on 90 mm diameter polystyrene Petri plates containing 25 mL of ger-

Table 1
Composition of media used for callus induction from anthers and ovaries of six grapevine cultivars. All media contained NN salts and MS vitamins and were solidified with 0.3% gelrite.

Medium	2,4-D (μM)	BA (μM)	Casein hydrolysate (% w/v)	Sucrose (% w/v)	pH
CIM-1	4.5	9.0	0.0	6.0	5.8
CIM-2	9.0	4.5	0.0	2.0	5.5
CIM-3	0.0	0.0	0.0	6.0	5.8
CIM-1A	4.5	9.0	0.1	6.0	5.8

mination medium consisting of CIM basal medium supplemented with 10 μM IAA, 1 μM gibberellic acid (GA3), 0.3% sucrose and 0.25% activated charcoal. The pH of the medium was adjusted to 5.8 with NaOH prior to autoclaving at 98 kPa and 121 °C, and medium was solidified using 0.3% gelrite (Duchefa). For plant conversion, germinated embryos with hypocotyls and cotyledons and an apical root axis were transferred to test tubes (25 mm × 120 mm) containing 25 mL of MS basal medium with half-strength macronutrients and supplemented with 0.15% sucrose. The pH of the medium was adjusted to 5.8 with NaOH prior to autoclaving at 98 kPa and 121 °C and medium was solidified using 0.8% agar. The cultures were maintained at 26 ± 1 °C (20 ± 1 °C night temperature) under a 16 h photoperiod with a photon flux density of 45 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

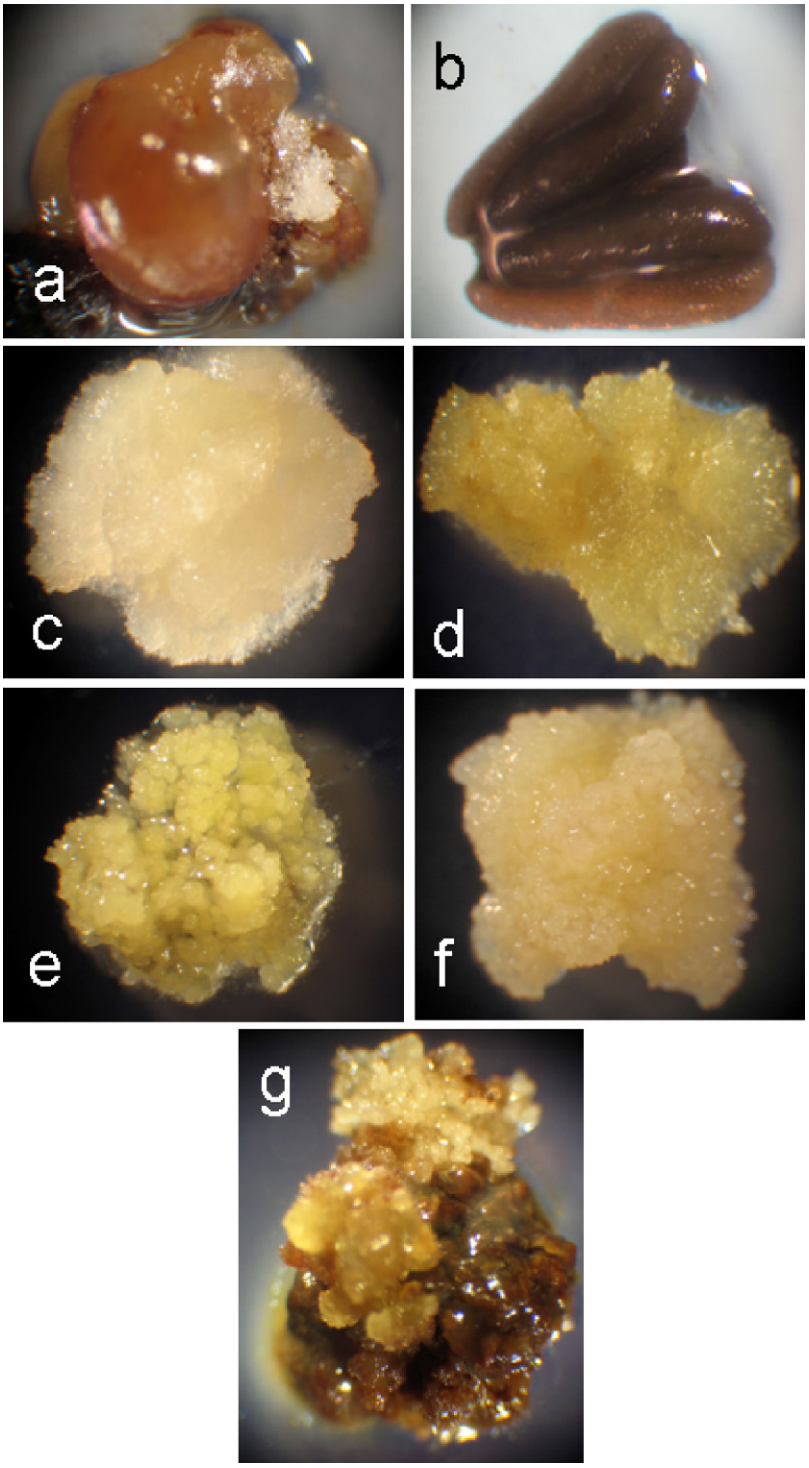

Fig. 2. Induction responses in anthers and ovaries of the grapevine. (a) Albariño ovary cultured in CIM-1 medium with little non-embryogenic callus formation. (b) Torrontés necrotic anther after culture in CIM-3 medium. (c) Non-embryogenic white callus formed from a Treixadura anther cultured in CIM-1 medium. (d) Non-embryogenic yellow callus formed from a Brancellao ovary cultured in CIM-1 medium. (e) Embryogenic yellow callus formed from a Merenzao anther cultured in CIM-1 medium. (f) Embryogenic whitish-yellow callus formed from a Merenzao anther cultured in CIM-1 medium. (g) Non-embryogenic brown and necrotic callus formed from a Brancellao anther cultured in CIM-1 medium.

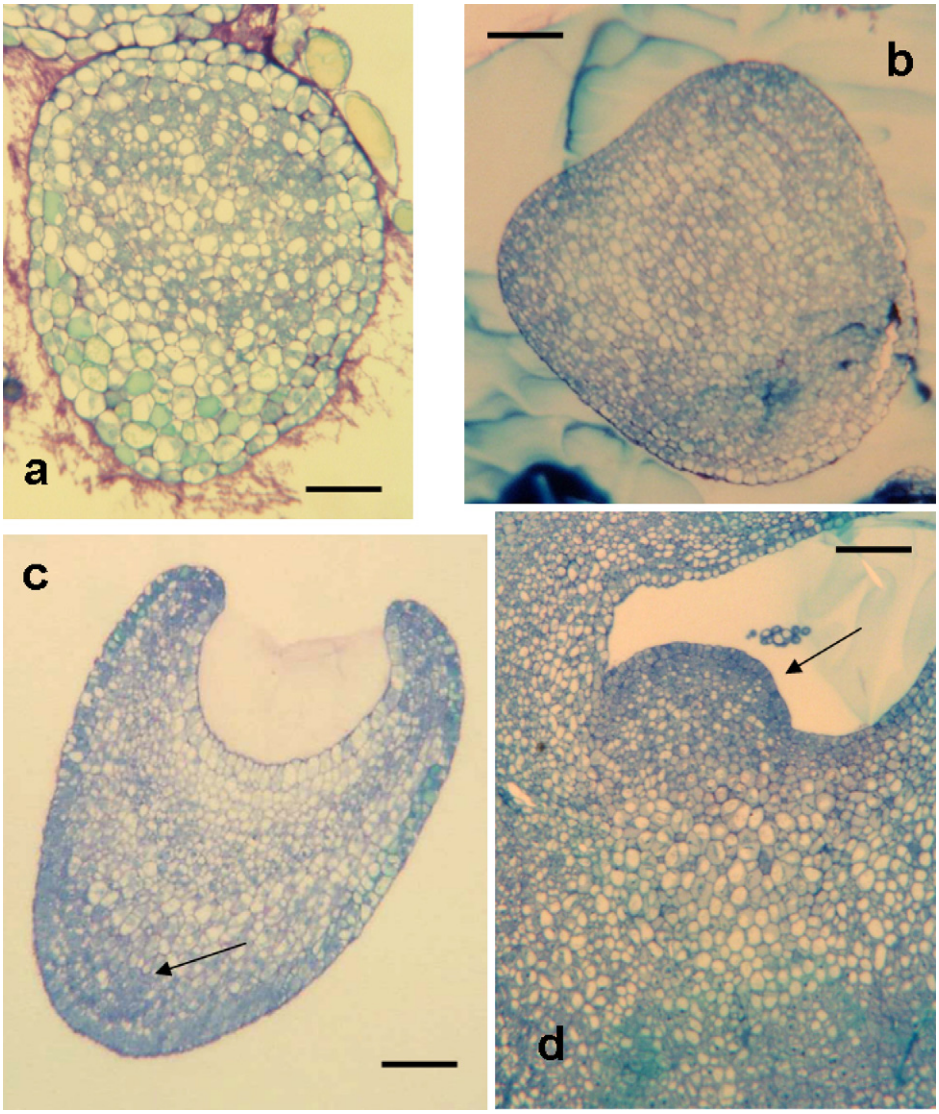

Fig. 3. Developmental stages of somatic embryos in embryogenic callus of grapevine after six months of culture in CIM-1 medium. (a) Globular embryo from Treixadura anther-derived embryogenic callus (bar = 50 μm). (b) Heart-shaped embryo from Mencía ovary-derived embryogenic callus (bar = 125 μm). (c) Torpedo-shaped embryo from Mencía anther-derived embryogenic callus showing the root meristem initials (arrow, bar = 125 μm). (d) Cotyledonary embryo from Mencía ovary-derived embryogenic callus showing a well developed shoot apical meristem (arrow, bar = 125 μm).

2.5. Histological analysis

Pieces of embryogenic callus were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), overnight, at 4 °C. After fixation, samples were dehydrated in acetone series and embedded in Technovit 8100 resin (Kulzer, Germany) at 4 °C. Blocks were polymerized at 4 °C and Technovit semithin sections (1 μm) were obtained and stained with 0.075% toluidine blue in water for 5 min for structural analysis. After rinsing and drying, preparations were mounted in Eukitt and observed under bright field in a Leitz Laborlux 12 microscope equipped with a DP10 Olympus digital camera.

2.6. Data analysis

A total of eight plates per cultivar, developmental stage and induction medium were used for anthers (25 explants per plate) and four plates were used for ovaries (10 explants per plate). To evaluate the effect of callus induction medium, the presence of somatic embryogenesis (embryogenic callus with or without visible embryos) on explants was recorded after 28 weeks in culture. Plates and explants that were lost due to contamination or death were not included in the statistical analysis. Data of percentages of somatic embryogenesis were statistically analyzed by the Kruskal–Wallis test at a significance level of $\alpha = 0.05$. Data on precocious germination in callus cultures in differentiation medium, as well as data on germination and plant conversion, were statistically analyzed using one-way ANOVA, and the obtained differences among means were contrasted using the Duncan multiple range test ($p = 0.05$). All statistical tests were performed using SPSS statistical software (version 17 for Windows, SPSS Inc., Chicago, IL).

3. Results

3.1. Induction of somatic embryogenesis

A total of 10,225 anthers and 2,050 ovaries were cultured in the induction media. Four embryo induction media were tested with anthers and ovaries of the six grapevine cultivars. No somatic embryogenesis induction was obtained using the medium CIM-3 in any explant of any cultivar. In all other tested media, both

Table 2
Average percentages of embryogenesis from anthers and ovaries of six autochthonous grapevine cultivars from Galicia collected at three different developmental stages (R1, R2 and R3). Means in each column (within genotype) or in each row (within culture medium, in parentheses) followed by the same letter are not significantly different as determined by a Kruskal–Wallis test ($p = 0.05$). NT: not tested.

R1 stage					R2 stage			R3 stage		
Cultivar and explant	Induction medium	No. of explants	No. with embryogenic response	% Embryogenesis (mean ± SE)	No. of explants	No. with embryogenic response	% Embryogenesis (mean ± SE)	No. of explants	No. with embryogenic response	% Embryogenesis (mean ± SE)
Albariño										
Anthers	CIM-1	200	1	0.50 ± 0.50 a (a)	187	0	0.00 ± 0.00 a (a)	200	0	0.00 ± 0.00 a (a)
	CIM-2	200	3	1.50 ± 0.96 a (a)	200	2	1.00 ± 1.00 a (a)	123	0	0.00 ± 0.00 a (a)
	CIM-1A	NT	–	–	185	9	4.67 ± 2.72 a (a)	130	4	2.77 ± 1.14 a (a)
Ovaries	CIM-1	40	2	5.00 ± 2.89 a (a)	40	0	0.00 ± 0 .00 a (a)	38	0	0.00 ± 0.00 a (a)
	CIM-2	40	5	12.50 ± 9.46 a (a)	40	5	12.50 ± 6.29 ab (a)	30	2	6.67 ± 6.67 ab (a)
	CIM-1A	NT	–	–	40	10	25.00 ± 2.89 b (a)	14	3	21.43 ± 1.67 b (a)
Treixadura										
Anthers	CIM-1	195	5	2.55 ± 0.95 a (a)	180	4	2.02 ± 1.17 a (a)	NT	–	–
	CIM-2	200	3	1.50 ± 0.95 a (a)	199	3	1.50 ± 0.96 a (a)	NT	–	–
	CIM-1A	NT	–	–	171	66	36.96 ± 5.28 c	NT	–	–
Ovaries	CIM-1	40	12	30.00 ± 5.77 b (b)	30	1	3.33 ± 3.33 a (a)	NT	–	–
	CIM-2	40	0	0.00 ± 0.00 a (a)	34	5	14.18 ± 4.79 ab (b)	NT	–	–
	CIM-1A	NT	–	–	40	8	20.00 ± 9.13 b	NT	–	–
Torrontés										
Anthers	CIM-1	197	2	1.00 ± 1.00 a (a)	199	0	0.00 ± 0.00 a (a)	198	0	0.00 ± 0.00 a (a)
	CIM-2	199	5	2.52 ± 0.97 a (a)	197	19	9.62 ± 0.90 b (b)	200	2	1.00 ± 1.00 a (a)
	CIM-1A	NT	–	–	158	2	1.05 ± 0.61 a (a)	105	0	0.00 ± 0.00 a (a)
Ovaries	CIM-1	40	0	0.00 ± 0.00 a (a)	40	4	10.00 ± 0.00 b (b)	38	0	0.00 ± 0.00 a (a)
	CIM-2	40	10	25.00 ± 6.46 b (a)	40	7	17.50 ± 4.79 c (a)	40	7	17.50 ± 2.50 b (a)
	CIM-1A	NT	–	–	30	2	6.34 ± 3.72 ab (a)	31	1	2.50 ± 2.50 a (a)
Mencía										
Anthers	CIM-1	198	5	2.55 ± 0.98 a (ab)	134	0	0.00 ± 0.00 a (a)	200	7	3.50 ± 0.96 a (b)
	CIM-2	195	7	3.55 ± 1.48 a (a)	94	0	0.00 ± 0.00 a (a)	NT	–	–
	CIM-1A	NT	–	–	NT	–	–	195	45	23.08 ± 0.51 b
Ovaries	CIM-1	40	0	0.00 ± 0.00 a (a)	39	0	0.00 ± 0.00 a (a)	40	0	0.00 ± 0.00 a (a)
	CIM-2	40	1	2.50 ± 2.50 a (a)	16	0	0.00 ± 0.00 a (a)	NT	–	–
	CIM-1A	NT	–	–	NT	–	–	40	7	17.50 ± 7.50 b
Brancellao										
Anthers	CIM-1	200	2	1.00 ± 1.00 a (a)	200	0	0.00 ± 0.00 a (a)	175	5	3.00 ± 0.58 a (b)
	CIM-2	175	0	0.00 ± 0.00 a (a)	199	0	0.00 ± 0.00 a (a)	197	2	1.02 ± 0.58 a (a)
	CIM-1A	168	11	6.24 ± 0.85 a (a)	173	1	1.33 ± 1.33 a (a)	44	1	2.63 ± 2.63 a (a)

Ovaries	CIM-1	40	0	0.00 ± 0.00 a (a)	40	0	0.00 ± 0.00 a (a)	40	8	20.00 ± 0.00 b (b)
	CIM-2	40	1	2.50 ± 2.50 a (a)	39	0	0.00 ± 0.00 a (a)	40	0	0.00 ± 0.00 a (a)
	CIM-1A	30	3	10.00 ± 10.00 a (a)	27	1	3.57 ± 3.57 a (a)	30	7	23.33 ± 8.82 b (a)
Merenzao										
Anthers	CIM-1	198	0	0.00 ± 0.00 a (a)	174	9	5.01 ± 1.72 a (b)	165	1	0.55 ± 0.55 a (ab)
	CIM-2	200	0	0.00 ± 0.00 a (a)	200	54	27.00 ± 2.52 b (b)	43	0	0.00 ± 0.00 a (a)
	CIM-1A	NT	–	–	145	46	26.25 ± 8.55 b (b)	34	0	0.00 ± 0.00 a (a)
Ovaries	CIM-1	30	1	3.33 ± 3.33 a (a)	40	1	2.50 ± 2.50 a (a)	37	0	0.00 ± 0.00 a (a)
	CIM-2	40	0	0.00 ± 0.00 a (a)	30	18	60.00 ± 5.77 c (b)	21	0	0.00 ± 0.00 a (a)
	CIM-1A	NT	–	–	37	10	27.03 ± 6.10 b (b)	23	0	0.00 ± 0.00 a (a)

anthers and ovaries produced embryogenic callus with or without visible embryos after 12 weeks of culture in all cultivars tested. They currently continue to grow by monthly transfer to the same induction medium, being periodically checked for the maintenance of their embryo differentiation ability. Increasing browning in the calli was observed with time of subculturing, making necessary manual selection of granular embryogenic calli for maintaining the cultures.

Four morphological responses were observed (Fig. 2). First, anthers or ovaries either did not grow or produced a small compact non-embryogenic callus (Fig. 2a and b); second, friable white or yellow non-embryogenic callus formed but did not develop further (Fig. 2c and d); third, embryogenic white or pale yellow granular callus formed with or without visible embryos (Fig. 2e and f); and fourth, brown and necrotic callus was observed (Fig. 2g).

Analysis at the light microscopy of samples containing the embryogenic callus and derived structures (Fig. 3) revealed the presence of somatic embryos in several developmental stages. Individual globular (Fig. 3a) and heart-shaped (Fig. 3b) embryos arose as independent structures without vascular connections among them or with the initial explant or derived callus; the embryos showed the typical protodermis or peripheral layer of polygonal cells. The root apex became to be recognizable at the basal pole in embryos at the torpedo stage, showing the initial cells of the root meristem (Fig. 3c). Cotyledonary embryos clearly showed a well formed shoot meristem at the apical pole (Fig. 3d). Meristems were characterized by the dense cytoplasms and big nuclei of their cells, which appeared darker in toluidine blue stained sections, in contrast with the clear and vacuolated cytoplasms of most embryo cells.

Embryogenic induction was influenced by the kind of starting explant, its developmental stage, the culture medium and the genotype (Table 2). For *V. vinifera* cultivar Albariño the developmental stage of the explants at the time of their collection did not significantly affect somatic embryogenesis, although the best results were obtained with explants collected at the late uninucleate pollen stage (R2), corresponding to stage VI according to Gribaudo et al. (2004).

Albariño anthers and ovaries formed embryogenic callus on several media (Table 2). Little to no response was observed in anthers and ovaries cultured on CIM-1 medium. The addition of casein hydrolysate (CIM-1A medium) increased the embryogenic responses from 0% to 4.67% and to 25% in R2 anthers and ovaries, respectively, and from 0% to 2.77% and to 21.43% in R3 anthers and ovaries, respectively. These results were better than those obtained using CIM-2 medium, which was somewhat effective in inducing embryogenic responses in ovaries independently of the collection date.

Treixadura ovaries collected at R1 performed better when cultured in CIM-1 medium (30%), but those collected at R2 performed better in CIM-2 medium (14.18%). Treixadura anthers, however, formed embryogenic callus at low frequencies independently of the collection stage. The results for explants collected at the R2 stage were significantly improved when both anthers and ovaries were cultured in the presence of casein hydrolysate (CIM-1A medium, Table 2), with 36.96% and 20% embryogenic responses, respectively.

Embryogenic responses in Torrontés anthers were low, performing significantly better in CIM-2 when collected at R2. Ovaries showed a higher embryogenic ability than anthers, and in the best medium (CIM-2) the embryogenic responses were independent of the collection stage, although the best percentage was observed for ovaries collected at R1.

Responses in Merenzao explants were practically restricted to those collected at R2 and formed embryogenic callus on all media tested. The best responses were obtained when explants were cul-

tured in CIM-2 medium, particularly with ovaries, which showed the highest responses for all cultivars in all culture combinations tested (60% embryogenesis). The use of casein hydrolysate (CIM-1A medium) did not improve the results for Merenzao explants.

Finally, for the cultivars Mencía and Brancellao the best results were obtained when explants were collected at the early binucleate pollen stage (R3). There were no differences in responses across types of explants for Mencía, and the results improved with the addition of casein hydrolysate. On the other hand, Brancellao ovaries performed better than anthers, both collected at the R3 stage, and were not improved with the addition of casein hydrolysate.

In summary, the best embryogenesis percentages were highly variable, showing that different explant/culture medium/flower development combinations are needed specifically for each cultivar tested. Therefore, the best combinations were as follows: for Albariño, ovaries collected at the R2 or R3 stages and cultured in the presence of casein hydrolysate (CIM-1A medium); for Treixadura, anthers collected at the R2 stage and cultured in CIM-1A medium, although explants at the R3 stage were not tested; for Torrontés, ovaries cultured in CIM-2 medium, independently of the developmental stage of the flowers at the time of collection; for Mencía, both explants collected at the R3 stage and cultured in CIM-1A medium; for Brancellao, ovaries at the R3 stage cultured in CIM-1 or CIM-1A media; and finally for Merenzao, ovaries at the R2 stage cultured in CIM-2 medium, although anthers also collected at the R2 stage and cultured in CIM-2 or CIM-1A media performed as well as the best responses obtained for the other cultivars.

The addition of casein hydrolysate, in the same medium, was beneficial in a general sense for all cultivars; however, it is remarkable that in some cases CIM-2 medium produced the best results (Torrontés explants at any developmental stage and Merenzao ovaries at R2 stage), since this medium contains the opposite phytohormonal balance and a third of the sucrose concentration compared with CIM-1 medium.

New embryogenic cultures are routinely initiated each year using the selected protocol for each specific cultivar. A detailed study of the influence of the collection year in the induction process was not performed, although we only observe variations in the contamination rates of the starting cultures, but not in the induction percentages obtained.

Table 3
Percentage of precocious germination in embryogenic calli cultured in DM differentiation medium. Means among cultivars are not statistically different at $p = 0.05$ after one way ANOVA. Data from Merenzao were excluded from the statistical analysis.

Cultivar	No. of calli	Total no. calli with precocious germination	% Precocious germination (mean ± SE)
Albariño	201	123	61.68 ± 6.06
Treixadura	226	79	38.76 ± 7.09
Torrontés	173	88	55.02 ± 9.98
Mencía	150	109	74.29 ± 8.60
Brancellao	23	13	58.93 ± 18.06
Merenzao	4	2	50.00

3.2. Embryo differentiation, germination and plant conversion

The transfer of embryogenic calli to DM differentiation medium initially induced the proliferation of the callus (Fig. 4a); on its surface embryos formed and subsequently proliferated by secondary embryogenesis, giving rise to clusters of somatic embryos at different developmental stages. After about two weeks of culture under these conditions, an extensive process of precocious germination started (Fig. 4b), which was detrimental to the proliferation of embryos by secondary embryogenesis. The presence of precocious germination was 56.45% on average and was not significantly different across the cultivars tested (Table 3). In light of these results, a second experiment was performed to test the ability of DM1 medium to possibly reduce precocious germination. The results obtained are presented in Table 4. Although the differences between media were not statistically significant, embryogenic calli cultured in DM1 medium showed a reduction in precocious germination of embryos, thus favoring embryo proliferation by secondary embryogenesis (Fig. 4c). In addition, the number of precociously germinating embryos per callus was also reduced (data not shown). Treixadura calli presented less precocious germination (Table 4) in both media tested (less than 5%), whereas Albariño presented the highest percentage of precocious germination in DM medium as

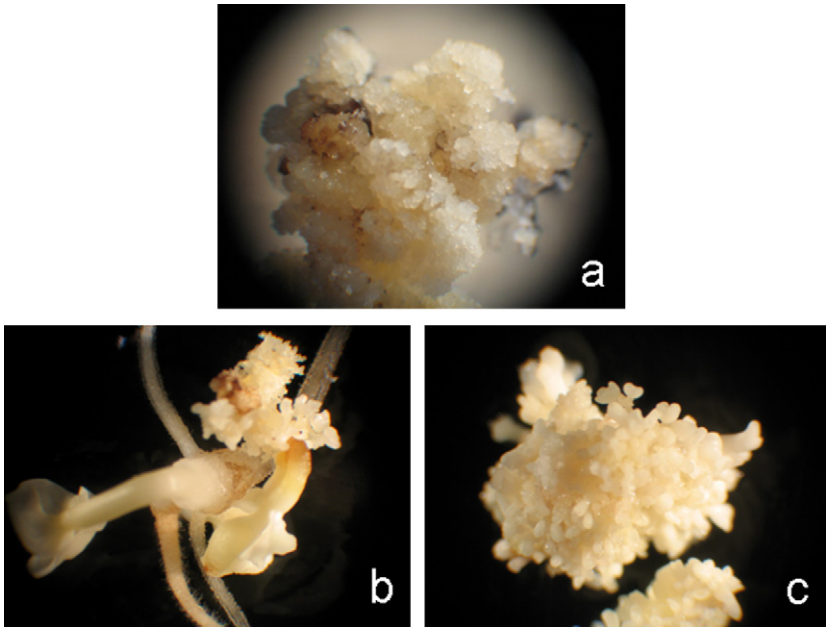

Fig. 4. Differentiation of somatic embryos in the grapevine cv. Merenzao. (a) Embryogenic callus from anthers after six months of culture in CIM-1A medium and 1 week of culture in DM differentiation medium. (b) Precocious embryo germination and secondary embryogenesis in a six-month old anther callus cultured in DM differentiation medium for one month. (c) Somatic embryo cluster proliferation in a six-month old anther callus cultured in DM1 differentiation medium for one month.

Table 4
Percentage of precocious germination in embryogenic calli cultured in two differentiation media. Means among cultivars (within media) followed by the same letter are not significantly different at $p = 0.05$ after one way ANOVA. NT: not tested.

Medium	Cultivar	No. of calli	Total no. calli with precocious germination	% Precocious germination (mean ± SE)
DM	Albariño	26	14	57.36 ± 14.71 b
	Treixadura	16	1	4.76 ± 4.76 a
	Torrontés	25	5	30.00 ± 15.27 ab
	Mencía	14	5	30.00 ± 15.27 ab
	Brancellao	15	5	29.53 ± 17.33 ab
	Merenzao	NT	–	–
				30.33 (average[a])
DM1	Albariño	26	2	7.79 ± 4.18 a
	Treixadura	16	0	0.00 ± 0.00 a
	Torrontés	25	7	33.81 ± 23.90 a
	Mencía	14	7	23.33 ± 23.33 a
	Brancellao	15	2	15.87 ± 9.65 a
	Merenzao	NT	–	–
				16.16 (average[a])

[a] Average values calculated over data of the five cultivars used for each differentiation medium.

well as the highest decrease in the occurrence of this phenomenon in DM1 medium. No significant differences between cultivars in DM1 medium were found.

Ungerminated embryos were transferred to germination medium, where germination rates were high for all cultivars tested (Table 5, Fig. 5). Germination rates for Albariño embryos were significantly lower ($p = 0.05$), however, than those obtained for all other cultivars. Mencía embryos more efficiently germinated under these conditions (97%). Germinated embryos were transferred to conversion medium for plant regeneration. In addition, embryos that precociously germinated in differentiation medium were also transferred to conversion medium directly.

Plant conversion was greatly affected by the origin of the germinated embryos (Table 6). On average, the conversion of embryos that precociously germinated early in the differentiation medium was 60.93%, whereas those that germinated normally in germination medium converted to plants at 85.94%. This difference was also observed on a per cultivar basis.

Considering the genotype of the embryos and taking into account that the number of Merenzao embryos tested was not enough for statistical analysis, there were statistical differences for the conversion of precociously germinated embryos, but not for the conversion of normally germinated embryos, suggesting that these later embryos are developmentally more uniform. The highest percentage of conversion of normally germinated embryos was obtained with Brancellao embryos, but these produced the lowest percentage when precociously germinated embryos were used.

Normal plantlets (Fig. 6a) were obtained both from normally and from precociously germinated embryos, but abnormal plants (Fig. 6b) with teratological symptoms and poor growth were also observed in both cases.

Table 5
Percentage of germination of isolated embryos in germination medium. Means followed by the same letter are not significantly different at $p = 0.05$ after one way ANOVA.

Cultivar	No. of embryos	Total no. germinated embryos	% Germination (mean ± SE)
Albariño	250	192	77.16 ± 6.27 a
Treixadura	174	143	84.58 ± 3.13 ab
Torrontés	257	232	91.12 ± 1.69 ab
Mencía	122	116	97.10 ± 1.86 b
Brancellao	270	231	90.19 ± 3.30 ab
Merenzao	30	29	93.33 ± 6.67 ab
			87.76 (average[a])

[a] Average value calculated over data of all cultivars.

Fig. 5. Germination of somatic embryos in grapevine cv. Treixadura after 1 month of culture in germination medium supplemented with 10 μM IAA, 1 μM GA3, 0.3% sucrose and 0.25% activated charcoal. Embryos originated from an anther callus induced in CIM-1 medium that was subsequently cultured in DM differentiation medium for 1 month before embryo isolation.

4. Discussion

Somatic embryogenesis induction was successfully obtained for all cultivars tested in this study with a simple protocol using NN-based media supplemented with only two combinations of 2,4-D and BA (Table 1). Differential induction responses were observed (Table 2) due to the interaction among the genotype of donor plants, the explant, its developmental stage in relation to the microsporogenesis as revealed by DAPI staining, and the culture medium composition.

This work further confirms the high effectiveness of 2,4-D and BA for somatic embryogenesis induction (Martinelli and Gribaudo, 2001; Perrin et al., 2004; López-Pérez et al., 2005; Pinto-Sintra, 2007; Oláh et al., 2009).These two growth regulators were also recently used by Vidal et al. (2009) for somatic embryogenesis induction from anthers and ovaries in eight grapevine cultivars, including Albariño, using an MS-based medium. It is interesting to note that for this cultivar our results were similar to theirs, but we were able to improve the induction from ovaries by inverting the

Table 6
Plant conversion from precociously and normally germinated embryos. Means among cultivars (within origin of embryos) followed by the same letter are not statistically different at $p = 0.05$ after one way ANOVA. Data from Merenzao were excluded from the statistical analysis.

Origin of germinated embryos	Cultivar	No. of embryos	Total no. of plants	% Conversion (mean ± S.E.)
Precocious	Albariño	161	96	60.78 ± 4.39 ab
	Treixadura	110	70	69.42 ± 6.59 b
	Torrontés	131	87	61.46 ± 9.36 ab
	Mencía	84	58	74.08 ± 6.34 b
	Brancellao	26	15	38.89 ± 24.22 a
	Merenzao	4	3	75.00
				60.93 (average[a])
Normal	Albariño	125	101	81.34 ± 3.84 a
	Treixadura	65	54	83.85 ± 7.73 a
	Torrontés	109	89	80.94 ± 5.97 a
	Mencía	82	67	89.87 ± 2.44 a
	Brancellao	74	66	93.72 ± 5.11 a
	Merenzao	8	6	75.00
				85.94 (average[a])

[a] Average values calculated over data of the five cultivars used for the statistical analysis for each type of embryo origin.

hormonal balance and adding casein hydrolysate, reaching up to 25% embryogenesis with ovaries collected at the R2 developmental stage (Table 2).

Reduced nitrogen sources are important for the development of embryogenic responses in the grapevine; ammonium is critical for maintaining cell viability and improving somatic embryo development and further plant conversion (Perrin et al., 2001). The use of additional reduced nitrogen organic sources, like glutamine or casein hydrolysate, has been reported in the literature as being beneficial to embryogenesis induction in the grapevine (Mauro et al., 1986; Martinelli et al., 1993; Kikkert et al., 2005). In particular, Kikkert et al. (2005) found that glutamine aided embryogenesis induction especially in ovaries. Vidal et al. (2009) reported similar results, since their NN-derived medium, which contained also glutamine, improved embryogenesis induction over medium lacking glutamine. In our experiments, we found that a more complex nitrogen source was the best medium for embryogenesis induction, with the exception of Merenzao and Torrontés (Table 2).

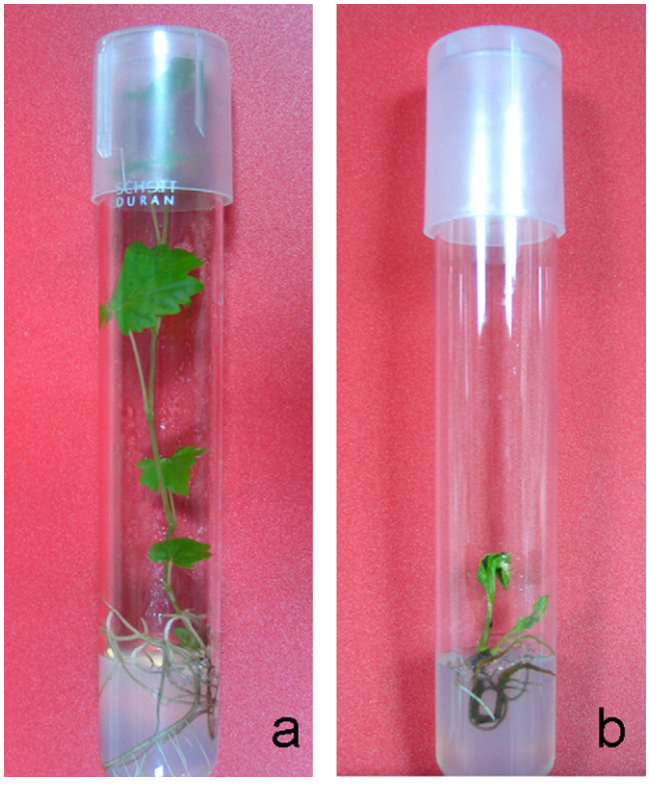

Fig. 6. Plant regeneration from somatic embryos in the grapevine cv. Mencía. (a) Normal plantlet obtained after 1 month of culture in conversion medium (MS basal medium with half-strength macronutrients without growth regulators and supplemented with 0.15% sucrose). (b) Abnormal plantlet obtained after 1 month of culture in conversion medium (MS basal medium with half-strength macronutrients without growth regulators and supplemented with 0.15% sucrose). Embryos were originated from an anther callus induced in CIM-1 medium and subsequently cultured in DM differentiation medium for 1 month before embryo isolation. Isolated embryos were cultured for 1 month in germination medium and then transferred to conversion medium.

The developmental stage of the explants at the time of their collection critically influences the efficiency of somatic embryogenesis induction (Gribaudo et al., 2004; Vidal et al., 2009). This influence is also dependent on the particular genotype of the cultivar being tested, as was also shown in the present study. It is interesting that this factor not only affects anthers, whose developmental stage is easy to identify by studying microsporogenesis with DAPI staining, but also appears to correlate with the behavior of other organs used as explants, like ovaries (Vidal et al., 2009; this study).

In this respect, it is also remarkable that the responses observed in this study were obtained at later stages of development than those observed by Gribaudo et al. (2004). In fact, the stages named R1 and R2 here correspond to stages V and VI identified by Gribaudo et al. (2004), but our R3 stage is later, corresponding to a microsporogenesis stage of binucleate microspores (Fig. 1). The best responses were obtained in our study using the R2 stage for four cultivars (Albariño, Treixadura, Torrontés, Merenzao). The other two cultivars (Mencía and Brancellao) presented the best responses at binucleate microspore stages (R3 stage).

On the other hand, Vidal et al. (2009) followed the guidelines proposed by Gribaudo et al. (2004) in their study, which included one of the cultivars tested here, Albariño. Our explants of this particular cultivar seemed to perform slightly better when collected at similar developmental stages. At least for Albariño, it is evident that the competence for somatic embryogenesis is retained for a long developmental period, with the best results being obtained at R2 stage. Even at R3 the best treatments allowed us to obtain an induction of somatic embryogenesis that was higher than 2.5% for anthers and 21% for ovaries. Hence, the embryogenic competence of these tissues is high, provided that a good protocol to express it is available. The question arises of which physiological and/or molecular factors are responsible for this competence. Elucidating these factors will undoubtedly improve the induction protocol for many genotypes and will render somatic embryogenesis a more reliable and efficient technology.

All cultivars tested presented precocious germination of embryos on callus and embryo clusters in DM differentiation medium (Table 4), which is probably due to precocious develop-

mental blockage of most initiated embryos (Perrin et al., 2001) and/or incorrect maturation and development. Albariño had the highest percentage of precocious germination and Treixadura the lowest. The use of DM1 differentiation medium greatly reduced this precocious germination, by half on average (Table 4), with different cultivars producing different results. Whereas on Torrontés and Mencía it had little to no effect, the medium greatly reduced precocious germination in Treixadura, Brancellao (by half) and, most significantly, Albariño. These results may reflect an interaction between exogenously applied hormones (in DM medium) and endogenous ones, as was already pointed out by Jiménez and Bangerth (2000); depending on the influence of genotypic features this interaction could produce abnormally developed, precociously germinating embryos. The use of a medium without hormones (DM1) could facilitate the establishment of a normal metabolism, producing mature and well developed embryos that will not germinate until transferred to germination medium. The hormones involved could include abscisic acid and indoleacetic acid (Faure et al., 1998); experiments are under way to elucidate the role that these hormones play in the control of grapevine somatic embryo development.

5. Conclusion

A complete protocol for normal plant regeneration from anthers and ovaries in six grapevine cultivars autochthonous from Galicia (Spain) was established. This is the first report for five of the cultivars (Treixadura, Torrontés, Mencía, Merenzao and Brancellao) and an improvement of the protocol for the sixth (Albariño). We were able to induce somatic embryogenesis in explants collected during the binucleate pollen microsporogenesis stage, which represents a wider window of competence in the initial explants. Detailed analysis of the occurrence of precocious embryo germination allowed us to select a differentiation medium without hormones that greatly reduces this phenomenon and increased secondary embryogenesis. Normally developed embryos germinated correctly at high rates when transferred to germination medium. Plant conversion was also higher when normal instead of precociously germinated embryos were used indicating the importance of embryo maturation and development. Our results confirm the importance of setting up a detailed protocol for somatic embryogenesis in the grapevine, due to the interaction among the genotype of donor plants, the explant, its developmental stage, and the culture medium composition.

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