Optimizing Initiation and Maintenance of *Vitis* Embryogenic Cultures

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Abstract. Stamens and pistils from mature grapevines and leaves from in vitro micropropagation cultures were used to optimize parameters influencing somatic embryogenesis in *Vitis*. Embryogenic competence was dependent on species/variety, explant type and developmental stage, medium composition, and growth regulator concentration. Of varieties evaluated, a greater number produced embryogenic cultures from stamens and pistils (26) compared with leaves (six). Among the different stamen and pistil stages, Stage II and III explants produced the maximum embryogenic response regardless of genotype and medium composition. Of seven culture media tested, the highest embryogenic response was recorded from varieties cultured on MSI (18) and PIV (16) media. Experiments annually repeated over 3 to 10 years demonstrated reproducible results. Highly reliable protocols for somatic embryogenesis were obtained for 29 *Vitis* species and varieties, including 18 *Vitis vinifera* varieties, *Vitis riparia*, *Vitis rupestris*, *Vitis champinii*, and eight *Vitis* hybrids. Embryogenic cultures were maintained on X6 medium for a period of 6 months to 2 years depending on the variety and used in studies involving genetic transformation and transgenic plant regeneration.

Genetic engineering of *Vitis* has emerged as an alternative to conventional breeding for the introduction of desirable traits into elite varieties. The routine use of embryogenic cultures for grapevine transformation (Gray et al., 2005) necessitates optimization of protocols for culture initiation and maintenance. Although somatic embryogenesis from *Vitis* was reported previously (Carmi et al., 2005; Gray and Mortensen, 1987; Kikkert et al., 2005; Perrin et al., 2001), few varieties displayed embryogenic competence and wide variations among responsive varieties were observed. To improve embryogenic competence and produce cultures that result in genetically stable regenerants, factors that influence somatic embryogenesis, including explant type and developmental stage, macro- and microelement composition of the culture medium and growth regulator concentration warrant exam-

ination. Many studies describing *Vitis* somatic embryogenesis used inflorescence tissues as explants for initiating embryogenic cultures (Carmi et al., 2005; Perrin et al., 2001, 2004). Grapevine flowering in most regions of the world occurs only once a year for few weeks, thereby providing a small window of opportunity to initiate embryogenic cultures. This makes it essential to identify the correct developmental stage of explants to optimize

embryogenic competence. A number of medium formulations are currently used for culture initiation for grapevine (Kikkert et al., 2005; Perrin et al., 2004). Identification

Fig. 1. Embryogenic culture system in *Vitis*. Unopened leaves (A) cultured on initiation medium produce sectors of embryogenic and nonembryogenic callus (B). (C) Stamens and pistil explants from unopened grapevine inflorescence. (Inset) A single stamen in an inverted position to show the filament connected to the anther. Callus production from filament tip (D) or connective tissue (E) of stamens. (F) Somatic embryo development and proliferation after transfer of embryogenic callus to X6 medium. (G) Germination of cotyledonary stage somatic embryo and plant regeneration (H). Scale bars: A = 1 mm; B, F = 5 mm; C, G = 0.5 mm; D, E = 0.1 mm.

Fig. 2. Stamen and pistil explant stages in *Vitis*. Four stages, Stage I (A–B), Stage II (C–D), Stage III (E–F), and Stage IV (G–H), were identified based on appearance of explants and development of microspores. Scale bars: A = 0.1 mm, C = 0.8 mm, E, G = 1.0 mm.

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of one to a few useful culture media would facilitate initiation of embryogenic cultures for *Vitis* species and varieties.

We studied factors including explant type and developmental stage, medium composition, and growth regulator concentrations repeatedly over a period of 3 to 10 years to optimize production of embryogenic cultures. Cultures were obtained in 29 *Vitis* species and varieties.

Materials and Methods

Influence of genotype and explant type

Embryogenic competence of in vitro-derived leaves, pistils, and stamens from 29 grape varieties, including 18 *Vitis vinifera* varieties, *Vitis champinii* Planch., *Vitis riparia* Michx., *Vitis rupestris* Scheele, and eight *Vitis* interspecific hybrids, was evaluated over a period of 10 years depending on the availability of explant material. Leaf explants of *V. vinifera* 'Autumn Seedless', 'Chardonnay', 'Cabernet Franc', 'Cabernet Sauvignon', 'Merlot', 'Pinot Noir', 'Semillon', 'Superior Seedless', 'Thompson Seedless', and 'White Riesling'; *V. rupestris* 'St. George'; and *Vitis* hybrids 'Blanc du Bois', 'Freedom', 'Seyval Blanc', and 'Tampa' were obtained from in vitro shoot cultures (Gray and Benton, 1991). Unopened leaves, 1.5 to 5.0 mm long (Fig. 1A), were placed abaxial side down on NB2 medium (Table 1). Five leaves were placed in each petri dish and incubated in darkness at 26 °C for 5 to 7 weeks. Resulting callus cultures (Fig. 1B) were transferred to cool white fluorescent light (65 $\mu\text{m}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 16-h photoperiod) at 26 °C for 5 weeks. Culture development was screened weekly and embryogenic callus was transferred to growth regulator-free X6 medium (Dhekney et al., 2008) for development of proembryonic masses (PEM) and somatic embryos (SE). Further PEM and SE proliferation occurred on X6 medium.

Inflorescences of 26 *Vitis* varieties (Table 2) were obtained either from the Mid-Florida Research and Education Center vineyard or from dormant vine cuttings obtained from the Foundation Plant Services, University of California, Davis, CA. Inflorescence development from dormant cuttings, 30 cm in length, was induced after surface sterilization in 25% NaClO solution with constant agitation for 5 min followed by two washes with sterile distilled water. Cuttings were forced to flower by placing them basal end down in 500-mL Erlenmeyer flasks containing 250 mL sterile distilled water to a growth room with cool-white fluorescent lights (65 $\mu\text{mole}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16-h photoperiod), at 26 °C for 3 weeks. Water was replaced and the basal 1 cm of each cutting was trimmed at weekly intervals. Inflorescences were excised and samples of individual flowers were dissected and observed with a stereomicroscope to determine the developmental stage of stamens and pistils (Fig. 1C). Inflorescences at varying developmental stages were surface-sterilized by immersion in 70% ethanol for 30 s followed by washing them in distilled water for

Table 1. Composition of media tested for embryogenic culture initiation in *Vitis*.

Medium ^z	Macro- and microelements	BAP (μM)	2,4-D (μM)	NOA (μM)	Sucrose (%)	Gelling agent and concn ($\text{g}\cdot\text{L}^{-1}$)	pH
MSI	MS	4.5	5.0	—	2.0	Agar (7.0)	6.0
MSII	MS	5.0	2.5	2.5	2.0	Agar (7.0)	6.0
X1 ^y	MS	5.0	2.5	2.5	2.0	Agar (7.0)	5.8
X2 ^y	MS	5.0	15.0	2.5	2.0	Agar (7.0)	5.8
PIV ^x	Nitsch	8.9	4.5	—	6.0	Phytigel (3.0)	5.7
NI	Nitsch	5.0	2.5	2.5	2.0	Agar (7.0)	5.8
NII	Nitsch	5.0	15.0	2.5	2.0	Agar (7.0)	5.8
NB2	Nitsch	1.0	5.0	—	2.0	Agar (7.0)	6.0

^zAll media were supplemented with 0.1 $\text{g}\cdot\text{L}^{-1}$ myo-inositol and MS vitamins.

^yX medium = modified MS medium lacking glycine and MS nitrate was replaced with X nitrate comprising of 3.033 $\text{g}\cdot\text{L}^{-1}$ KNO_3 and 0.364 $\text{g}\cdot\text{L}^{-1}$ NH_4Cl (as the sole nitrogen source).

^xPIV medium (Franks et al., 1998) was supplemented with B5 vitamins instead of MS vitamins pH was adjusted with KOH before autoclaving.

Table 2. Embryogenic response of *Vitis* stamens and pistil explants (Stage I to IV).

Variety	Stage	No. of stamens cultured	Percent embryogenic stamens <i>V. vinifera</i>	No. of pistils cultured	Percent embryogenic pistils
Barbera (3) ^z	I	35	0	5	0
	II	280	0	40	2.5
	III	210	0	30	0
	IV	1,960	0.05	280	1.1
Cabernet Franc (7)	I	245	2.4	35	2.0
	II	1,855	2.5	265	5.3
	III	8,330	1.4	1,190	5.3
	IV	2,345	0.4	335	1.2
Cabernet Sauvignon (10)	I	3,185	0.1	455	0.2
	II	23,730	0.06	3,390	0.5
	III	15,505	0.4	2,215	1.7
	IV	5,425	0.1	775	1.3
Chardonnay (8)	I	595	1.8	85	3.5
	II	6,545	1.5	935	3.3
	III	3,885	1.4	555	3.4
	IV	1,470	0.7	210	4.8
Dolcetto (3)	I	1,050	0.1	150	0
	II	1,855	0	265	0
	III	2,730	0	390	0
	IV	2,100	0	300	0
Emerald Seedless (3)	I	945	1.3	135	0
	II	805	3.5	115	0.9
	III	225	7.3	175	0
	IV	595	2.9	85	1.2
Merlot (9)	I	980	5.4	140	0.0
	II	5,775	9.9	825	9.3
	III	5,495	11.6	785	13.8
	IV	2,170	3.0	310	3.9
Pinotage (3)	I	525	0	75	0
	II	2,590	0	370	0.5
	III	3,080	0.03	440	0.2
	IV	315	0	45	0
Pinot Gris (3)	I	175	1.7	25	4
	II	1,785	0.7	255	0
	III	1,470	0.07	210	0
	IV	70	1.4	10	0
Pinot Noir (3)	I	350	1.4	50	2
	II	4,935	3.1	705	0.6
	III	3,745	1.1	535	0.6
	IV	3,850	1.2	550	2.0
Sauvignon Blanc (5)	I	385	0.2	55	0
	II	4,235	0.1	605	3.8
	III	1,225	0.2	175	2.9
	IV	245	1.2	35	17.1
Shiraz (5)	I	140	0	20	0
	II	2,485	0.6	355	0.3
	III	1,715	0.5	245	0
	IV	140	1.4	20	0
Thompson Seedless (5)	I	1,260	9.5	180	0.6
	II	1,050	10.5	150	4
	III	1,120	9.9	160	3.1
	IV	420	6.2	60	8.3

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Table 2. (Continued) Embryogenic response of *Vitis* stamens and pistil explants (Stage I to IV).

Variety	Stage	No. of stamens cultured	Percent embryogenic stamens <i>V. vinifera</i>	No. of pistils cultured	Percent embryogenic pistils
White Riesling (10)	I	8,890	1.1	1,270	1.4
	II	14,490	2.7	2,070	3.6
	III	12,040	3.7	1,720	5.9
	IV	3,605	2.1	515	4.3
Zinfandel (6)	I	630	0	90	1.1
	II	455	0	65	1.5
	III	2,590	0.4	370	3.0
	IV	2,310	0.04	330	0
<i>V. champinii</i> (3)	I	350	0	50	0
	II	490	0.2	95	0
	III	700	1.85	100	0
	IV	280	0	40	0
<i>V. riparia</i> Gloire (3)	I	245	0	35	0
	II	1,330	0.2	190	1.1
	III	3,570	0.5	510	2.4
	IV	455	0.9	65	4.6
<i>V. rupestris</i> St. George (3)	I	315	0	45	0
	II	595	1.5	85	0
	III	595	1.0	85	1.2
	IV	385	0	55	0
<i>Vitis</i> hybrids					
Blanc du Bois (7)	I	2,065	0.4	295	0
	II	3,710	0.3	530	0
	III	6,860	0.2	980	0
	IV	1,820	0.2	260	0
Conquistador (5)	I	350	0	50	0
	II	105	0	15	0
	III	1,295	0.84	185	0.54
	IV	805	0	115	1.7
Freedom (5)	I	385	0	55	0
	II	980	0	140	0
	III	595	0	85	1.2
	IV	735	0	105	5.7
Harmony (3)	I	280	0	40	0
	II	350	0.57	50	0
	III	1,085	0.82	155	0
	IV	315	0	45	0
Richter 110 (4)	I	35	0	5	0
	II	2,065	0	295	0.7
	III	2,590	0.1	370	0
	IV	770	0	110	0
Seyval Blanc (5)	I	245	0	35	0
	II	245	3.3	35	0
	III	1,085	0.3	155	1.9
	IV	1,435	0	205	2.0
Stover (5)	I	1,505	0.1	215	0.5
	II	5,355	0.4	765	2.6
	III	5,495	0.2	785	1.7
	IV	1,925	0.4	275	2.2
Tampa (3)	I	280	0	40	0
	II	840	0	120	0.8
	III	350	0	50	0.8
	IV	280	1.8	40	0
SE			0.2		0.3
Heterogeneity χ^2 results			df	χ^2	<i>P</i> value
Variety $\times$ stage (percent responsive stamens)			75	40.84	0.522
Variety $\times$ stage (percent embryogenic pistils)			75	133.31	<0.001

Data were analyzed using heterogeneity χ^2 test for independence. SE values for percent embryogenic stamen and pistil explants are included at the end of the table. Data represents the cumulative response of explants on 8 culture media.

*Numbers in parenthesis indicate the number of years cultures were initiated to study embryogenic response from anther and stamen explants.

30 s. Inflorescences were then washed in 25% NaClO solution containing one drop Triton X-100 with constant agitation for 5 min followed by three 5-min washes in sterile distilled water. Stamens (anthers with intact filaments) were carefully separated from the calyptra and the pistil before placing them on the medium. The pistil with the remaining filament stubs also

was recovered and placed on the medium. Each petri dish contained 35 stamens in a clump in the center along with the pistils from which they were obtained placed near the perimeter. Petri dishes were sealed with Parafilm® (Fisher Scientific International, Inc., Hampton, NH) and placed in darkness at 26 °C. Four floral stages were recognized based on the size of

inflorescences and individual flowers, anther size, and anther color (Fig. 2):

Stage I. Flower clusters were ≈ 2.5 to 3.0 cm long, individual flower buds 0.5 to 0.7 mm in diameter, and anthers 0.1 to 0.2 mm in length, white in color and clear in appearance (Fig. 2A–B). Anthers were fragile and extremely difficult to observe at this stage. Microspores were single-celled and densely cytoplasmic with thin cell walls when mounted in 4% mannitol.

Stage II. Flower clusters were ≈ 6 to 8 cm long and individual flower buds ≈ 1.5 mm in diameter. Anthers were 0.8 to 1.0 mm long, yellowish in color, and appeared translucent with clear walls (Fig. 2C–D). The locule was cloudy and white in color. Microspores were yellowish in color and densely cytoplasmic with a thickening wall.

Stage III. Flower clusters were ≈ 9 to 10 cm long and individual flower buds were 1.5 to 2.0 mm in diameter. Anthers were 1.0 mm in length, yellowish in color, and cloudy in appearance with clear walls (Fig. 2E–F). The locule was cloudy and yellowish in color. Microspore walls were thicker and well developed.

Stage IV. Flower clusters greater than 10 cm in length and individual flower diameter was similar to Stage III. Anthers were 1.0 mm long and yellowish in color (Fig. 2G–H) with completely opaque walls. The locule was yellow in color and opaque. Microspore walls were thicker and pores in the cell wall were evident. The number of stamens and pistils cultured from each developmental stage varied according to the availability of flower buds at the particular stage.

After 4 weeks, cultures were transferred to cool-white fluorescent lights ($65 \mu\text{mole}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16-h photoperiod). Developing cultures were examined using a microscope for the presence of embryogenic callus at weekly intervals for 16 weeks.

Influence of culture medium on embryogenic competence

The influence of eight medium formulations on embryogenic competence was examined (Table 1). Embryogenic response of leaves was tested on NB2 medium while the embryogenic response of stamens and pistils was compared on the remaining media. Most media were supplemented with 2% sucrose and pH-adjusted with 1 N KOH before the addition of $7 \text{ g}\cdot\text{L}^{-1}$ TC agar (Phytotechnology Laboratories, LLC, Shawnee Mission, KS; Catalog No. A 175) with the exception of PIV medium (Franks et al., 1998), which was supplemented with 6% sucrose and $3 \text{ g}\cdot\text{L}^{-1}$ Phytigel® (Sigma-Aldrich Co., St. Louis, MO). All media were autoclaved at 120 °C and $1.1 \text{ kg}\cdot\text{cm}^{-2}$ for 20 min and 25 mL medium was dispensed into each $100 \times 15\text{-mm}$ petri dish.

Maintenance of embryogenic cultures

The development of embryogenic callus from a stamen or pistil was recorded as a positive response with calluses being transferred to X6 medium for PEM and SE development. PEM and SE were transferred

to fresh X6 medium at 4- to 6-week intervals as previously described (Li et al., 2008). After the first transfer, 0.2 g of PEM from each variety was transferred to fresh X6 medium. The number of cotyledonary-stage SE was recorded after 6 weeks to evaluate differences in embryogenic competence. There were three petri dishes for each variety, each comprising a replication. The experiment was conducted three times.

Somatic embryo germination and plant regeneration

Cotyledonary stage SE from *Vitis* genotypes were induced to germinate on MS1B medium (Dhekney et al., 2008). Five SE were placed in each petri dish, which was replicated three times. Enlarged cotyledons were excised after 2 weeks to permit faster shoot growth and development (Li et al., 2008). The number of plants developing from each variety was recorded. Plants with a robust shoot and root system were subsequently transferred to Magenta M-7 boxes (Fisher Scientific International, Inc., Hampton, NH) containing 30 mL MS medium for further development. Well-developed plants were acclimatized by placing them in transparent plastic domes under cool-white fluorescent lights ($65 \mu\text{mole}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16-h photoperiod) at 26°C for 3 weeks and then transferred to a greenhouse.

Statistical analysis

Categorical, count, and percentage data were analyzed using heterogeneity χ^2 testing for independence (Compton, 2005; Zarr, 1984). Treatment means of variables with a significant χ^2 ($\alpha \leq 0.05$) were compared using the sample SE (Mize et al., 1999). Data for plant recovery from germinated SE were analyzed using Proc GLM and analysis of variance procedures of Statistical Analysis Systems (2001).

Results and Discussion

Influence of genotype and explant type. Leaf explants cultured on NB2 medium produced sectors of compact, cream-colored embryogenic callus and loose, brown-colored nonembryogenic callus (Fig. 1B). Of 15 varieties tested, only six produced embryogenic callus (Fig. 3). The highest percentage of embryogenic competent explants was recorded in 'Superior Seedless' (72%) and 'Thompson Seedless' (53%). Transfer of embryogenic callus to growth regulator-free X6 medium resulted in the production of somatic embryos. Leaves from all three seedless varieties evaluated produced embryogenic cultures. At this point, however, it is unknown if seedlessness could be a potential factor influencing embryogenesis from leaf explants because seeded *V. rupestris* 'St. George' and complex interspecific hybrids 'Freedom' and 'Seyval Blanc' also responded. More seedless varieties should be evaluated before a definitive relationship can be established.

Fig. 3. Embryogenic response of *Vitis* leaf explants on NB2 medium. Five explants were placed in each Petridish containing 25 mL NB2 medium with three replicates. Embryogenic response was recorded after 10 to 12 weeks. Means with the same letter are not statistically significant according to standard error of means (SEM = 8.1).

Table 3. Embryogenic response of *Vitis* varieties on seven media treatments.

Variety	Medium ^z	No. of stamens cultured	Percent embryogenic stamens	No. of pistils cultured	Percent embryogenic pistils
<i>Vitis vinifera</i>					
Barbera (3) ^y	X1	1,155	0.09	165	1.2
	X2	1,330	0	190	1.1
Cabernet Franc (7)	MSI	5,110	2.4	730	7.3
	PIV	1,400	0.6	200	6.0
	X1	3,325	0.3	475	2.3
	X2	3,115	2.1	445	4.5
Cabernet Sauvignon (10)	MSI	10,080	0.1	1,440	1.6
	MSII	5,005	0.06	715	1.7
	PIV	4,060	0.2	580	1.9
	X1	9,205	0.05	1,315	0.3
	X2	8,960	0.5	1,280	0.08
	MSI	1,680	1.3	240	3.3
Chardonnay (8)	MSII	1,680	2.8	240	6.3
	NI	770	0.5	110	6.4
	NII	735	0.4	105	1.0
	PIV	980	1.8	145	3.4
	X1	910	4.4	130	4.6
	X2	1,260	1.1	180	2.8
	X1	3,955	0.03	565	0
	MSI	3,570	4.3	510	0.4
Merlot (9)	MSI	3,995	10.8	265	35.1
	MSII	2,065	7.9	295	7.8
	NI	350	8.1	50	6
	NII	385	4.7	55	0
	PIV	1,575	17.6	225	24
	X1	665	9.6	95	4.2
	X2	700	3.4	100	2.0
	MSI	2,450	4.4	350	12.9
Orange Muscat (4)	MSII	840	5.5	120	13.3
	PIV	910	2.4	130	17.7
	MSI	4,725	0.02	675	0.3
Pinotage (3)	PIV	1,785	0	255	0.4
	MSI	3,080	0.03	440	0.2
Pinot Gris (3)	MSI	2,485	2.8	355	1.1
	MSII	2,730	3.5	390	0.8
Pinot Noir (6)	PIV	805	0.2	115	0.9
	MSI	1,260	0.08	180	2.8
Sauvignon Blanc (5)	MSII	1,435	0.7	205	4.4
	NI	245	0	35	5.7
	NII	245	0	35	5.7
	PIV	1,505	0	215	4.2
	X1	315	0.3	45	4.4

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Table 3. (Continued) Embryogenic response of *Vitis* varieties on seven media treatments.

Variety	Medium ^z	No. of stamens cultured	Percent embryogenic stamens	No. of pistils cultured	Percent embryogenic pistils
Shiraz (5)	MSI	4,165	0.6	595	0.2
Thompson Seedless (7)	MSI	1,610	9.8	230	3.9
	MSII	1,435	11.6	205	3.4
	NI	875	4.8	125	0.8
	PIV	280	27.1	40	10
White Riesling (10)	MSI	6,055	5.5	865	5.1
	MSII	2,100	5.9	300	8.3
	NI	1,365	2.2	195	2.6
	NII	1,260	0.2	180	0.6
	PIV	1,155	5.5	165	4.8
	X1	7,770	3.8	1,110	7.6
	X2	4,095	1.4	585	3.9
Zinfandel (6)	X1	3,010	0.2	430	0.5
<i>V. champinii</i> (3)	MSI	1,750	0.80	250	0
<i>V. riparia</i> Gloire (3)	MSI	4,550	0.5	650	1.7
	PIV	805	0.1	115	5.2
	X2	2,975	0.2	425	2.6
<i>V. rupestris</i> St.George (3)	X1	1,190	0.8	170	0
	X2	1,365	0.4	195	0.5
	NI	700	0.42	100	0
<i>Vitis</i> hybrids					
Blanc du Bois (7)	X1	5,040	0.2	720	0
	X2	4,585	0.1	655	0
	N I	1,115	0.1	165	0
	N II	1,225	0.2	175	0
Conquistador (5)	MSI	1,295	0.69	161	0
	NI	105	1	15	0
	NII	70	1.4	10	0
	PIV	175	0	25	4.0
Freedom (5)	MSI	770	0	110	5.5
	MSII	805	0	115	0.9
Harmony (3)	MSI	2,030	0.54	290	0
Richter 110 (4)	MSI	4,585	0.02	655	0.3
	PIV	875	0.1	125	0
Seyval Blanc (5)	MSI	525	0	75	1.3
	MSII	350	0	50	4.0
	NI	245	0	35	2.
	NII	175	0.6	25	0
	PIV	490	2.0	70	0
	X1	210	0	30	10.0
Stover (5)	X1	4,655	0	665	0.3
	X2	4,725	0.02	675	0.4
	NI	1,365	0.5	195	1.0
	NII	1,365	2.19	195	16.4
	PIV	805	0.1	115	5.2
Tampa (3)	MSII	280	0	40	2.5
	PIV	595	0.8	85	1.2
SE			0.5		0.6
Heterogeneity χ^2 results			DF	χ^2	P value
Variety $\times$ medium (percent responsive stamens)			60	163.26	<0.001
Variety $\times$ medium (percent embryogenic pistils)			60	423.18	<0.001

^zData for only those medium treatments that produced an embryogenic response from either stamen or pistil explants has been provided. Data were analyzed using heterogeneity χ^2 test for independence. SE values for percent embryogenic stamen and pistil explants are included at the end of the table. Data represents the cumulative response of four developmental stages of explants.

^yNumbers in parentheses indicate the number of years cultures were initiated to study embryogenic response from anther and stamen explants.

Embryogenic response from stamens and pistils at four developmental stages was studied. Several reports of grapevine embryogenesis describe explants as anthers and ovaries (Carmi et al., 2005; Perrin et al., 2001, 2004). We consider this to be an incorrect designation of explants, because entire stamens and pistils are excised from developing inflorescences and cultured. The embryogenic response of stamens and pistils was variety-dependent. Among 26 varieties tested, a greater number produced embryogenic cultures from stamens (25) com-

pared with pistils (22) after 16 to 18 weeks (Table 2). These results contrast earlier studies (Kikkert et al., 2005) in which ovaries were found to be more responsive. Callus production from stamen explants occurred almost exclusively from the filament tip or the connective tissue (Fig. 1D–E). In most cases, the anthers failed to callus, turning brown during the culture period (Fig. 1E). Somatic embryos and subsequent plant regeneration most probably occurred from somatic tissues rather than microspores in as much as earlier studies using cytological and

genetic evidence (Bouquet et al., 1982; Rajasekaran and Mullins, 1983) demonstrated a somatic origin of calli and subsequent plant regeneration. Because embryogenic cultures arose exclusively from somatic tissues including the filament and anther wall, it was unnecessary to stain anthers and determine the developmental stage of microspores as suggested in previous studies (Hollo and Misik, 2000; Krastanova et al., 2000). The culture of intact stamens (anthers with attached filament) was essential for obtaining an embryogenic response. No response was observed from stamen explants with damaged filaments or when anthers were detached from filaments and cultured separately (data not shown). Similarly, detached filament explants failed to respond (data not shown). These results contrast studies (Perrin et al., 2004) in which wounding of anthers led to a greater embryogenic response. Although embryogenic cultures were obtained from all four developmental stages of explants in some varieties, more varieties as well as a greater percentage of explants produced cultures at Stage II and III of development. Compared with the number of varieties producing embryogenic cultures from Stage I (13) and IV (15) explants, more varieties were responsive from Stage II (18) and Stage III (21) explants. Although no significant differences were observed among stamen explants ($P = 0.522$), pistil explants at different stages of development differed significantly in their embryogenic response ($P < 0.001$). Among the four explant stages cultured, the highest embryogenic response among varieties was observed when Stage II and III explants were used (Table 2). Similarly, the percentage of explants responding from Stage II and Stage III were greater compared with the other stages. Earlier reports of grape somatic embryogenesis from anthers suggested a higher embryogenic response when translucent green–yellow anthers were cultured (Goussard et al., 1991; Newton and Goussard, 1990); however, a detailed description of developmental stages was not provided. Because grapevine flowering occurs only once a year in most regions and provides a short timeframe for culture initiation, a complete description of explant stages along with photographic evidence (Fig. 2) should serve as a comprehensive guideline for successful culture initiation. Among the varieties evaluated, the highest embryogenic response was observed from ‘Merlot’ stamens and pistils ($11.6\% \pm 0.2\%$ and $13.8\% \pm 0.3\%$) followed by ‘Thompson Seedless’ ($10.5\% \pm 0.2\%$ and $8.3\% \pm 0.3\%$) and ‘White Riesling’ ($3.7\% \pm 0.2\%$ and $5.9\% \pm 0.3\%$), respectively.

Embryogenic culture initiation on seven medium treatments. A significant difference in embryogenic response was obtained from both stamens and pistils ($P < 0.001$) on seven medium treatments (Table 3). Embryogenic cultures from stamens were established from the maximum number of varieties on MSI medium (18) followed by PIV (16), X1 (11), and MSII (11). A similar response was observed from pistils cultured on these media.

Among the varieties studied, the highest percentage of stamens producing embryogenic callus was recorded in 'Thompson Seedless' on PIV medium ($27.1\% \pm 0.5\%$), whereas 'Merlot' pistils produced the greatest response ($35.1\% \pm 0.6\%$) on MSI medium. Embryogenic cultures were obtained from explants of all 26 varieties tested. MSI medium comprises of MS macro- and microelements (Murashige and Skoog, 1962) and has a higher macroelement concentration compared with PIV medium (Franks et al., 1998), which is comprised of Nitsch macro- and microelements (Nitsch and Nitsch, 1969). PIV medium, however, has higher levels of growth regulators ($8.9 \mu\text{M}$ BAP and $4.5 \mu\text{M}$ 2,4-D) compared with MSI medium ($4.5 \mu\text{M}$ 2,4-D and $5.0 \mu\text{M}$ BAP). Other varieties responded solely on X1 medium, which is characterized by significantly higher inorganic nitrogen levels compared with MSI and PIV media. Thus, embryogenic competence appears to result from a complex interaction of several factors, including variety, macro- and micronutrient levels, and growth regulator concentration. Earlier studies involving grape embryogenesis used various media and growth regulator combinations to produce embryogenic cultures (Iocco et al., 2001; Kikkert et al., 2005; Perrin et al., 2004); however, the basal salts used in these media also were either MS or Nitsch.

Maintenance of embryogenic cultures.

Embryogenic callus obtained from leaf, stamen, and pistil explants produced somatic embryos after transfer to X6 medium. Cultures growing on X6 medium typically were comprised of PEM along with SE at the globular, heart, torpedo, and cotyledonary stages of development (Fig. 1F). Embryogenic cultures were maintained by careful transfer of proliferating PEM to fresh X6 medium at 4- to 6-week intervals. To evaluate differences in embryogenic competence among varieties, the number of cotyledonary stage SE produced from 0.2 g PEM was recorded after 6 weeks on X6 medium. Production of SE (embryogenic competence) on X6 medium was variety-dependent (Fig. 4). The maximum number of SE was produced from 'Thompson Seedless' (927) followed by 'Merlot' (575) and 'Shiraz' (569). X6 medium is characterized by higher levels of inorganic nitrogen, NH_4Cl as a source of NH_4 ion, and the presence of activated charcoal (Dhekney et al., 2008; Li et al., 2008). Grapevine SE proliferate by direct secondary embryogenesis with new embryos emerging from epidermal or subepidermal cells (Gray, 1995; Jayasankar et al., 2003). Thus, cultures could be maintained for a long period of time on this medium by careful transfer of PEM to fresh medium at 4- to 6-week intervals (Gray, 1995). In contrast to previous reports (Kikkert et al., 2005; Perrin et al., 2004), X6 medium was optimum for maintenance of embryogenic cultures of all varieties regardless of the medium from which they were initiated. Use of a single medium greatly increases the ease of long-term culture main-

Fig. 4. Embryogenic competence of *Vitis* varieties. The number of cotyledonary stage somatic embryo obtained from 0.2 g proliferating proembryonic masses after 6 weeks of culture on X6 medium was recorded as the embryogenic potential of a variety. Means with the same letter are not statistically significant according to standard error of means (SEM = 44).

Fig. 5. Plant recovery from germinated somatic embryo (SE) of *Vitis* varieties 'Cabernet Franc' (CF), 'Dolcetto' (DO), 'Harmony' (HM), 'Merlot' (MT), 'Ramsey' (RS), 'Riparia Gloire' (RS), 'Richter 110' (R-110), 'Seyval Blanc' (SB), 'Shiraz' (SH), 'Thompson Seedless' (TS), 'White Riesling' (WR), and 'Zinfandel' (ZF). Five cotyledonary stage SE were placed in a petri dish containing 25 mL MS1B medium with three replicates. Statistical analysis was carried using Proc GLM and analysis of variance procedures of SAS. Bars represented by the same letter are not significantly different according to Student-Newman-Keuls test ($\alpha = 0.05$).

tenance after initiation. An important factor affecting maintenance of embryogenic cultures was the use of TC agar as a gelling agent. Use of other gelling agents, including Bactoagar and Phytigel, in X6 medium resulted in a rapid decline in embryogenic competence and eventual termination of cultures (unpublished results). Use of TC agar or an equivalent type is therefore a critical factor for ensuring successful long-term maintenance of embryogenic cultures.

Somatic embryo germination and plant regeneration. SE germinated after transfer to MS1B medium (Fig. 1G). Plant recovery from germinated SE was variety-dependent (Fig. 5). Among the 11 varieties evaluated, the maximum plant recovery from germinated SE was observed for 'Seyval Blanc' (86.7%) followed by 'Thompson Seedless' and 'Merlot' (73.3% each). Plants with a shoot and root system were transferred to

Magenta M-7 boxes (Fig. 1H) and successfully established in a greenhouse after acclimatization. Several treatments have been used to improve grapevine SE germination (Gray, 1989; Jayasankar et al., 2005); however, cotyledon excision of germinated SE is a simple method resulting in greater plant recovery (Li et al., 2008).

Embryogenic cultures now are used routinely in *Vitis* for production of transgenic plants with desirable traits (Dhekney et al., 2008, 2009; Li et al., 2001, 2006, 2008). The protocols described here can assist in rapid initiation and establishment of cultures for use in genetic transformation studies.

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