PN-ABY-453
99046 10.278

FINAL REPORT

Covering Period: 8/21/90 to 8/20/95 (includes no-cost extension)

Submitted to Human Capacity Development
Bureau for Global Programs, Field Support and Research
U.S. Agency for International Development

SOMATIC EMBRYOS FOR GERMPLASM STORAGE AND CLONAL PROPAGATION

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Project Number 10.278
Grant Number: DHR-5600-G-00-0057-00

A.I.D. Grant Project Officer: Dr. Joel Cohen

Project Duration: 8/21/90 to 8/20/95 (includes no-cost extension)

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Executive Summary:

The purpose of this project was to develop alternative methods of germplasm conservation and propagation for crops such as grape and coffee. Genotypes, including cultivars, of these crops currently can be conserved only as plants growing in a plantation or vineyard setting. This method of conservation is so expensive as to have stifled conservation of these crops as well as others like them. Preserving both the genetic diversity and the genetically-fixed phenotypes of such crops is of paramount importance in order to insure an adequate genetic base for future improvement and to meet changing environmental stresses. Successful implementation of this project would eliminate the primary expense of germplasm conservation by allowing cultivars to be maintained as clonal somatic embryos (instead of intact plants), which then could be kept more efficiently in conventional seed germplasm repositories. Many other crops and native species cannot currently be conserved for similar reasons; this represents a critical problem as native plants are destroyed due to population growth.

During this project, we advanced toward the goal of storable somatic embryos by accomplishing the following: 1) We increased the number of genotypes from which useful cultues can be recovered. 2) We developed a highly-refined culture system for initiating and manipulating embryos. 3) We refined methods to control embryo maturation and plant recovery. 4) We evaluated various methods to dehydrate and/or cryopreserve embryos for prolonged storage. These improvements have moved us closer to a useful system. As a direct result of this project, a significant literature base has emerged that both has disseminated this new information and has effectively increased the awareness and promoted the idea of clonal germplasm conservation to a wide international audience. This latter conclusion is based on the numerous contacts and reprint requests recieved from developing countries and the emergence of similar research goals, as judged from surveying the literature and reviewing grant proposals, which cite our AID-supported publications.

A) Research Objectives: The purpose of this project was to develop technology in order to utilize somatic embryos as "synthetic seeds" for clonal germplasm conservation and propagation of two model species (coffee and grape). This project is of strategic importance to insure the conservation of most clonally-propagated species, by drammatically decreasing the cost of long-term maintenance. Many of these species already are being lost due to population growth and destruction of natural environments in conjunction with near total lack of adequate funds to accomplish conventional conservation. Research in this specific area or related areas of technology are underway worldwide. Recently, an entire book was devoted to the subject of using somatic embryos as synthetic seeds (Redenbaugh, 1993) that contains an overwhelming documentation of similar research being conducted by others. The book also contained two chapters (Gray and Compton, 1993; Gray et al., 1993) that resulted from and were directly supported by this project. This specific project was not supported by other organizations.

B) Methods and Results: Accomplishments are separated based on crop type (grape or coffee). Published or accepted manuscripts are referenced and attached to this report.

Grape:

For grape, emphasis was placed on: 1) development of methods to produce embryogenic cultures from additional species and varieties, 2) refinement of culture conditions to optimize growth of embryogenic cultures, and 3) evaluation of culture pretreatments to cause better embryo maturation and desiccation tolerance.

Development of methods to produce embryogenic cultures:

The ability to produce embryogenic cultures from many grape species and varieties has been limited by apparent genotypic specificities. Of approximately 30 described species in the genus *Vitis*, embryogenesis has been reported in only three: *V. longii*, *V. rupestris* and *V. vinifera*, or hybrids between these and other species. It is fortunate that *V. vinifera* is responsive, since it is the major grape of commerce. Genotypic specificity also exists within these species, since only certain varieties will respond.

Our methodology to induce and maintain embryogenic cultures of *V. rotundifolia* (muscadine grape) is presented in the attached AID-supported publication (Gray, 1992). Thus, this report extends the embryogenic response to a new species, *V. rotundifolia*, which is grown extensively in the Southeastern US and contains approximately 100 named varieties. Varieties are variously used for fresh fruit, juice, jelly and wine production. Our approach has been to utilize a combination of ovule and embryo culture to induce embryogenic cultures. Although resulting cultures arise from zygotic embryos and not desirable clonal tissue, this methodology demonstrates that embryogenesis is possible in this genus. We refined the methodology and demonstrate the technique for additional varieties. Reports describing this research were published (Gray, 1992; Gray and Hanger, 1993). Both papers acknowledge A.I.D. funding.

Other research to develop embryogenic culture systems:

Leaves from in vitro micropropagation cultures of *V. rotundifolia* varieties mentioned above and *V. vinifera* 'Cabernet Sauvignon', 'Grenache', 'Ruby Cabernet' and 'Thompson Seedless' were cultured on Nitsche's medium with 5 μM 2,4-D and 1 μM BA in attempts to induce embryogenic cultures. To date, we have found that 10 percent of 'Thompson Seedless' leaves produce embryogenic cultures and a single embryogenic culture of 'Cabernet Sauvignon' has been recovered.

We were able to produce embryogenic cultures from leaves of *V. rotundifolia* 'Carlos' by utilizing a unique three-step culture system. In this system, leaves obtained from micropropagation cultures were first cultured on Nitsche's medium with 5 μM 2,4-D and 1 μM BA for four weeks, then transferred to medium with 5 μM NAA and 1 μM BA for four weeks prior to transfer to basal medium. Apparently, the second medium promotes cell divisions, resulting in the survival of more embryogenic cells.

The three-step medium culture system was tested on additional germplasm. We first developed a cultivar collection of potted stock plants that could be maintained in the greenhouse. These stock plants then were utilized as source material for in vitro micropropagation cultures. Cultures of 15 cultivars were established, leaves of which were utilized as explants in somatic embryo initiation experiments. Methodology for obtaining these cultures will be published (Compton & Gray, 1994; Myerson et al., 1994). Table 1 lists the micropropagation cultures that were be used.

Subsequently, leaves from established cultures were placed into the three-step culture system. The following cultivars produced embryogenic cultures in this system: Carlos, Dixie, Autumn Seedless, Cabernet Sauvignon, Thompson Seedless and Niagara Seedless.

Optimization of media for growth of perennial embryogenic cultures:

In instances where maintenance of distinct cell lines is difficult, reliable culture initiation procedures are essential in order to utilize the embryogenic response at all. Perennial embryogenic culture systems (ie. those that can be maintained for prolonged time periods of up to several years), in which embryogenic cells are self-renewing, allow long-term maintenance of elite embryogenic lines as well as increased flexibility in choice of approaches to genetic manipulation. Examples of perennial embryogenic culture systems include carrot (*Daucus carota* L.), orchardgrass (*Dactylis glomerata* L.) and several grape (*Vitis* spp.) species and cultivars.

We previously described a method for maintenance of perennial cultures through direct secondary embryogenesis on media devoid of growth regulators. Somatic embryos tended to form at the hypocotyl/radicle boundary of previously-developed embryos; these could be separated and transferred to fresh medium to produce more embryos. Embryogenic cultures of *Vitis* hybrid 'Seyval Blanc' developed by Krul & Worley (J. Amer. Soc. Hort. Sci., 1977, 102:360-363) were maintained via this method for over 10 years.

Table 1. In vitro micropropagation cultures of grape developed during 1993 to be used as explants in embryogenic culture initiation.

Species	Cultivars
Vitis bourginiana	Black Spanish
Vitis champini	Dog Ridge
Vitis rotundifolia	Carlos
	Dixie
	Southern Home
Vitis vinifera	Autumn Seedless
	Cabernet Sauvignon
	Carignane
	French Colombard
	Lambrusco
	Thompson Seedless
	Tokay
Vitis hybrids	Himrod
	Niagra Seedless
	Blanc du Bois

We developed a new system, which was first described recently (Gray, 1992) for perennial embryogenic culture maintenance. Cultures of *Vitis longii*, whereby embryogenic cell clusters isolated from the growth regulator-induced sectoring type of callus, proliferated for at least nine years by recurrent subculture on medium devoid of growth regulators (unpublished). During 1993, we conducted experiments to describe optimization of the culture system for *Vitis vinifera* L. 'Thompson Seedless' and identify some important parameters that influence it.

Embryogenic cultures of *V. vinifera* 'Thompson Seedless' were initiated from leaves obtained from actively-growing in vitro adventitious shoot cultures, which were prepared and maintained as previously described for *Vitis* hybrids 'Orlando Seedless' and 'Tampa' (Gray & Klein, 1988). Embryogenic callus was isolated from explant tissue and placed (5 calli per petri dish) on growth regulator-free solidified MS medium. The MS medium was experimentally modified and eventually optimized as described below. Embryogenic callus was selectively transferred to fresh medium every 6 weeks. During transfer, larger somatic embryos and disorganized or necrotic callus tissues were discarded and only finely divided, watery, clear-to-white material, sometimes containing numerous globular embryos and often located at the base of larger embryos, was transferred. This material was typically scarce in newly-isolated lines such that

the production of several cultures were often combined for subcultures of approximately 3 mm in diameter. The relative amount of embryogenic material gradually increased through repeated selection to yield cultures that could be easily subdivided and rapidly increased.

A succession of experiments were conducted to develop optimized culture conditions. Preliminary experiments were used to determine general parameters that then were investigated further. Culture conditions that were optimized experimentally included, effect of activated charcoal, type and concentration of nitrogenous compounds and sucrose concentration.

Table 2 shows that MS medium modified to contain either 13.5 mM NH_4 and 15 mM NO_3 or 6.8 mM NH_4 and 30 mM NO_3 was superior to all others tested with respect to dry weight gain. Conventional MS medium, which contains the equivalent of 27 mM NH_4 and 30 mM NO_3 was noticeably inferior. Differences in dry weight gain are a clear indicator for differences in growth rate. Over the six-week culture cycle, after which measurements were taken, cultures on the two optimized ammonium-nitrate media had grown five-to-nine times as much as those grown on MS media (Tables 2-7).

Table 2. Effect of ammonium and nitrate on milligram dry weight increase of embryogenic *Vitis vinifera* 'Thompson Seedless' cultures.

	mM KNO_3				
mM NH_4Cl	0	7.5	15	30*	60
0	0.5fg[1]	1.6ef	1.0fg	1.1fg	0.3fgh
6.8	0.5fg	3.5cd	3.4cd	7.0a	2.7de
13.5	0.5fg	4.6bc	6.2a	5.0b	0.1fgh
27*	0.0g	1.0fg	3.6bcd	0.8fg*	0.4fgh
54	0.0g	0.0g	0.6fg	0.5fg	0.0g

* Denotes levels of ammonium and nitrate in standard MS medium.
[1] Values within or between columns followed by the same letter are not significantly different at $P \leq 0.05$ according to Student Newman Keul's test.

Table 3. Effect of ammonium and nitrate on number of embryos per callus in *Vitis vinifera* 'Thompson Seedless' cultures.

	mM KNO_3				
mM NH_4Cl	0	7.5	15	30*	60
0	0b[1]	0.9b	1.4b	0.2b	0.1b
6.8	0b	0b	0.1b	4.7a	0b
13.5	0b	0b	6.3a	1.4b	0b
27*	0b	0b	0b	0.1b*	0b
54	0b	0b	0b	0b	0b

* Denotes levels of ammonium and nitrate in standard MS medium.
[1] Values within or between columns followed by the same letter are not significantly different at $P \leq 0.05$ according to Student Newman Keul's test.

Similar to dry weight, the media modified with either 13.5 mM NH_4 and 15 mM NO_3 or 6.8 mM NH_4 and 30 mM NO_3 was superior to all others tested with respect the number of embryos produced.

Table 4. Effect of ammonium and nitrate on callus browning rating in *Vitis vinifera* 'Thompson Seedless' cultures. 1 = no browning; 2 = ¼ of culture surface brown; 3 = ½ of culture surface brown; 3 = 3/4 of culture surface brown; 4 = culture uniformly brown.

	mM KNO_3				
mM NH_4Cl	0	7.5	15	30*	60
0	4a[1]	4a	4a	4a	4a
6.8	1.4e	1f	1f	2d	2.7c
13.5	2d	1f	1f	2d	3b
27*	2d	2d	1f	3b*	3b
54	2d	3b	2d	2d	3b

* Denotes levels of ammonium and nitrate in standard MS medium.
[1] Values within or between columns followed by the same letter are not significantly different at $P \leq 0.05$ according to Student Newman Keul's test.

A number of ammonium-nitrate concentrations exhibited reduced browning (Table 4). These included combinations that were shown to be superior in dry weight gain and embryo production.

Table 5. Comparison of optimized ammonium and nitrate supplied in MS medium as either NH_4Cl and KNO_3 (experimental salts utilized for data in tables 2-4) or as NH_4NO_3 and KNO_3 (salts used in MS medium) on fresh and dry weight gain.

Salt Combination	Fresh Weight (mg)	Dry Weight (mg)
$NH_4Cl + KNO_3$	175a[1]	17a
$NH_4NO_3 + KNO_3$	201a	18a

[1] Values within or between columns followed by the same letter are not significantly different at $P \leq 0.05$ according to Student Newman Keul's test.

Comparison of the experimental salts (utilized in order to be able to formulate desired ratios tested) with reformulated medium using normal MS salts showed no significant difference in response. This demonstrated that comparative results obtained in tables 2-4 were valid in testing ammonium-nitrate relationships in comparison to MS medium and that the improved ratio could be reformulated using traditional MS salts.

Table 6. Effect of charcoal concentration in optimized medium on fresh and dry weight gain in embryogenic *Vitis vinifera* 'Thompson Seedless' cultures.

Charcoal (g/l)	Fresh Weight Gain (mg)	Dry Weight Gain (mg)
0	204b[1]	12b
0.5	273a	16a
1.0	223b	13b
2.0	218b	12b

[1] Values within or between columns followed by the same letter are not significantly different at $P \leq 0.05$ according to Student Newman Keul's test.

Charcoal added to medium at 0.5 g/l produced significantly more growth than either no charcoal or 1 - 2 g/l. Perhaps the beneficial effects of charcoal are due to its filtering effect, whereby culture exudates that normally are toxic to long-term growth are become inactivated.

Table 7. Effect of sucrose concentration in optimized medium on fresh and dry weight gain in embryogenic *Vitis vinifera* 'Thompson Seedless' cultures.

Sucrose (%)	Fresh Weight Gain (mg)	Dry Weight Gain (mg)
0	5d	0c
3	156c	19b
6	313b	21b
9	343a	30a

[1] Values within or between columns followed by the same letter are not significantly different at $P \leq 0.05$ according to Student Newman Keul's test.

Table 7 illustrates that the highest level of sucrose tested (9%) resulted in the best culture growth. Reformulation of MS culture medium to contain optimized levels of ammonium and nitrate with 0.5 g/l charcoal and 9% sucrose has resulted in cultures that grow approximately ten times faster when compared to equivalent cultures grown on "standard" MS media.

Pretreatments to enhance germination and desiccation tolerance of grape somatic embryos:

A number of different pretreatments have been tested to date. Treatment with abscisic acid (ABA), which has been shown to increase or induce desiccation tolerance in alfalfa and carrot, was shown to be ineffective in grape. We conducted a series of factorial experiments that tested ABA from concentrations of 1-160 μm and found no beneficial effect on either embryo germination percentage or ability to survive dehydration. It is known that grape somatic embryos contain endogenous ABA at levels high enough to cause dormancy to occur. It may be that the somatic embryos are physiologically saturated with endogenously-produced ABA such that additional ABA does not cause a response.

Therefore, treatment with abscisic acid (ABA), which increased or induced desiccation tolerance in alfalfa and carrot, was shown to be ineffective in grape. We explored the effect of the polyamine inhibitor MGBG. The effects of sucrose, ABA and MGBG, which were previously discussed in the 1992 annual report, were published in an article that acknowledges AID support (Compton & Gray, 1993).

Growth of cultures on elevated sucrose and/or inositol concentrations has provided the best results to date. Our normal maintenance medium utilized 6% sucrose. Embryos germinate precociously when cultures are transferred to lower levels of sucrose. When cultures are placed on medium with increased levels of sucrose (9 and 12%) or on medium with 1% inositol, more embryos of select morphology (ie. embryos with two cotyledons and narrowed hypocotyls that closely resemble zygotic embryos) are produced. We found that the number of high-quality embryos increases as cultures are maintained over time (up to 18 weeks) on media with elevated sucrose.

When select embryos from these media are germinated and scored for development into plants, we found that those from the elevated sucrose levels produced more plants than those from control or inositol treatments (Table 8). With grape, the primary difficulty in obtaining plants from somatic embryos has been the failure of the shoot apex to develop. Our results suggest that embryos maintained on these higher sucrose levels grow in a more normal manner, with better shoot apical meristem development.

Table 8. The number of plants obtained from *Vitis vinifera* 'Thompson Seedless' somatic embryos pretreated at different sucrose and inositol concentrations for 18 weeks.[1]

Sucrose (g/l)	Inositol (g/l)	Number of embryos	Number of plants	Percent plant recovery
60[2]	1	4	0	0
60	10	16	1	6
90	1	20	7	35
120	1	27	5	19
150	1	6	4	67

[1]Somatic embryos were germinated on MS medium with 1 μm BA.

[2]Normal somatic embryo maintenance medium.

Ongoing pretreatment experimentation is designed to evaluate the effects of various growth inhibitors, such as ancymidol and tetcyclasis, on embryo quality and desiccation tolerance.

Effect of ABA on somatic embryo germination, secondary embryo production and plant formation:

The effect of ABA on somatic embryogenesis was examined by subculturing somatic embryos representing all developmental stages to medium (MS with 412.5 mg/l NH_4NO_3 and 475 mg/l KNO_3, 1 g/l myo-inositol, 120 g/l sucrose and 0.7% TC agar at pH 5.4; SE) containing either 0, 1, 10 or 100 μM ABA. ABA was filter-sterilized and added to cooled, autoclaved medium. Culture vessels were 100 x 15 mm plastic petri dishes that contained approximately 50 ml of medium. Cotyledonary stage embryos were laid individually on the medium surface. There were 4 plates per treatment with 16 embryos per plate. Torpedo stage embryos were cultured in groups of 10 with 5 groups per plate, whereas heart and globular stage embryos were cultured in clumps weighing approximately 15 mg. There were 4 plates per treatment each containing 5 clumps. Data on precocious embryo germination, embryo viability (as determined by the ability to stain pink after tetrazolium treatment) and the number of secondary embryos produced were recorded for each cotyledonary and torpedo stage embryo after 4 weeks. The number of plants obtained from each treatment was recorded after 4 weeks (8 weeks after ABA treatment) on germination medium (MS with 3% sucrose, 1 g/l myo-inositol, 1 μM BA

and 0.7% agar). Data recorded for heart and globular stage embryos included the number of cotyledon, torpedo, heart and globular stage embryos produced after 4 weeks.

The addition of 10 and 100 μM ABA to SE medium reduced precocious germination, the percentage of embryos that produced secondary embryos and the number of secondary embryos of cotyledonary and torpedo stage embryos without reducing their viability (Table 9). The ability of cotyledonary embryos to development into plants was reduced following the 4 week incubation on medium that contained ABA, whereas the ability of torpedo stage embryos to develop into plants was approximately doubled following incubation on medium with 1 μM ABA compared to embryos incubated on medium lacking ABA. Plant formation from torpedo stage embryos was reduced following incubation on medium with 10 or 100 μM ABA.

ABA dramatically affected the development of heart and globular stage embryos (Table 10). At 1 μM ABA the percentage of cotyledonary and torpedo stage embryos formed after 4 weeks was approximately 6 to 29 percent greater than the controls, whereas the percentage of heart and globular embryos was reduced 7 to 24 and the total number of embryos reduced 29 to 38 percent over the control. Higher ABA concentration significantly inhibited the development of cotyledonary stage embryos but did not severely reduced the formation of torpedo stage embryos. This suggests that embryo maturation was promoted at 1 μM ABA, as determined by the higher percentage of cotyledonary and torpedo stage embryos, whereas total embryo production was reduced.

Table 9. Somatic embryo germination, secondary embryo production and plant formation from cotyledonary and torpedo stage somatic embryos of 'Thompson Seedless' grape treated with various concentrations of ABA.

Embryo stage[1]	ABA conc (μM)	Precocious germination (%)	Embryo viability[2] (%)	Secondary embryo production			Plant development[5]	
				(%)[3]	Number[4]		(%)	
C	0	98	100	50	3.6	± 0.8	13	(4/32)
	1	89	100	56	3.7	± 0.7	3	(1/32)
	10	0	88	25	0.4	± 0.1	0	(0/32)
	100	0	100	39	0.9	± 0.2	0	(0/32)
T	0	64	100	85	9.5	± 2.6	6	(5/80)
	1	81	100	80	4.7	± 1.1	11	(9/80)
	10	0	100	65	1.5	± 0.4	0	(0/80)
	100	0	90	75	2.0	± 0.5	1	(1/80)

[1] There were 64 (cotyledonary) and 80 (torpedo) embryos per treatment.

[2] Embryo viability was estimated by incubating embryos in tetrazolium overnight. Sixteen and 20 cotyledon and torpedo stage embryos, respectively, were examined.

[3] The percent of embryos that formed secondary somatic embryos.

[4] Number of secondary embryos per somatic embryo.

[5] Embryos were transferred to modified MS medium with 1 μM BA for germination and plant development.
± values represent the standard error of the mean.

Table 10. Effect of ABA on the development of cotyledonary and torpedo stage embryos from heart and globular stage somatic embryos of 'Thompson Seedless' grape.[1]

Embryo stage	ABA conc (μM)	No. embryos[2]	Embryo developmental stages								
			Cotyledonary			Torpedo			Heart / Globular		
			%[3]		No.[4]	%		No.	%		No.
H	0	1268	8.8	(100)	111	38.2	(100)	485	53.0	(100)	672
	1	901	10.4	(118)	94	49.1	(129)	442	40.5	(76)	365
	10	644	0	(0)	0	54.0	(141)	348	46.0	(87)	296
	100	596	0	(0)	0	69.0	(181)	411	31.0	(58)	185
G	0	2272	4.3	(100)	98	44.6	(100)	1013	51.1	(100)	1161
	1	1409	5.1	(118)	72	47.3	(106)	666	47.6	(93)	671
	10	729	0.4	(9)	3	35.5	(75)	259	64.1	(125)	467
	100	612	0	(0)	0	52.5	(118)	321	47.5	(93)	291

[1] The initial weight of the cultures was approximately 15 mg at the beginning of the experiment.

[2] Total number of somatic embryos after 4 weeks.

[3] Percent of the total number of embryos.

[4] The number of somatic embryos after 4 weeks.

Values in () represent the percent change from the control.

Effect of methylglyoxal bis-(guanylhydrazone) (MGBG) on somatic embryogenesis:

The effect of MGBG on grape somatic embryogenesis was examined by subculturing embryogenic cells to SE medium containing MGBG at 0, 0.1, 1 or 10 mM concentrations. MGBG was filter-sterilized and added to cooled, autoclaved medium. Culture vessels consisted of 100 x 15 mm plastic petri plates with approximately 50 ml of medium. Embryogenic cells were cultured in clumps (5 per plate) that weighed approximately 15 mg and subcultured to fresh medium monthly. There were 5 plates per treatment. Each month the fresh weight of each culture and the number of cotyledonary embryos were recorded. The dry weight of each plate was recorded at the end of the experiment (3 months).

Previous studies have shown that the production of abnormal grape somatic embryos may be related to an increase in endogenous polyamine synthesis during the torpedo stage (Faure et al., 1991). MGBG inhibits the synthesis of two polyamines, spermidine and spermine, by blocking the decarboxylation of S-adenosylmethionine (SAM) (Galston and Kaur-Sawhney, 1987) and its addition to SE medium may improve the quality of grape somatic embryos by inhibiting polyamine synthesis. The addition of 1 to 10 mM MGBG to grape SE medium inhibited culture growth 15 to 100 percent during the 3 month culture period (Table 11). Growth inhibition was less prevalent during the first month but became more apparent after the first subculture and resulted in the death of cells incubated at 10 mM MGBG by the third month. The production of normal (zygotic appearing) embryos was completely inhibited by the addition of 10 mM MGBG but was not reduced by the addition of 0.1 or 1 mM MGBG during the first month of culture. However, a significant reduction in the number of normal cotyledonary embryos occurred when embryogenic cultures were incubated on SE medium with 1 mM MGBG for more than 1 month. No difference in the number of cotyledonary embryos was detected between embryogenic cultures incubated on medium with 0 or 0.1 mM MGBG through the course of the experiment.

Polyamine and ethylene synthesis are interrelated (i.e., ethylene production elevates when polyamine synthesis is blocked and visa versa) (Galston and Kaur-Sawhney, 1987) and the addition of MGBG has been shown to increase ethylene synthesis while inhibiting polyamine production (Roberts et al., 1984). Therefore, it is not known at present if the growth inhibition of grape embryogenic cells is caused by the addition of MGBG or by a possible increase in endogenous ethylene production.

Table 11. Effect of methylglyoxal bis-(guanylhydrazone) (MGBG) on the growth and development of somatic embryogenic cell cultures of 'Thompson Seedless' grape.

MGBG conc (mM)	Culture fresh weight (mg)				Culture dry weight (mg)				Number of cotyledonary embryos	
					1 month					
0	1809	±	54	(100)	-				3.0	± 1.6
0.1	1769	±	199	(97)	-				4.4	± 1.3
1	1528	±	218	(84)	-				5.2	± 1.0
10	850	±	26	(47)	-				0	
					2 months					
0	1673	±	69	(100)	-				2.6	± 1.1
0.1	1844	±	82	(110)	-				4.8	± 1.9
1	1429	±	37	(85)	-				0.8	± 0.4
10	319	±	26	(19)	-				0	
					3 months					
0	1676	±	280	(100)	374	±	40	(100)	3.3	± 0.9
0.1	1946	±	234	(116)	439	±	35	(116)	3.4	± 0.9
1	471	±	56	(28)	190	±	10	(51)	0.4	± 0.2
10	0				0				0	

± values represent the standard error of the mean. Values in () represent the percent change from the control.

Dehydration experiments:

Dehydration experiments have been conducted continuously during the span of this project. We have tested the response of embryos pretreated with ABA and have evaluated various parameters of our dehydration system, including the effects of dehydration time, relative humidity and rehydration methods. This work is detailed in the attached publication "Grape somatic embryo dormancy and quiescence: potential of dehydrated synthetic seeds for germplasm conservation", which is a book chapter in "Synseeds: Applications of synthetic seeds to crop improvement", CRC Press (Gray and Compton, 1993). This chapter acknowledges AID support.

In general, we have not been able to produce embryos that are adequately able to survive dehydration, although, pretreatment with sucrose has provided the best results to date. Preliminary dehydration experiments have been conducted on embryos from these treatments. It appears that embryos from the 12% sucrose treatments are able to survive dehydration better than those from lower sucrose concentrations, as determined by greening and enlargement of rehydrated embryos, although we have not yet achieved acceptable survival rates.

Cryopreservation experiments:

Embryogenic cultures of *Vitis vinifera* 'Thompson Seedless' were provided to Dr. Dufour and Ms. Abdelnour at CATIE in order to test their cryopreservation protocol (described below for coffee) on grape. This work was initiated in February 1992 at CATIE, but did not succeed.

Coffee

Research with coffee has concentrated on both dehydration methodology and cryopreservation. The cultivars 'Robusta 3751' and 'Catimor MP-547' were utilized due to their responsiveness in producing embryogenic cultures. Cultures are initiated by placing 1.5 cm^2 leaf sections on induction medium. Somatic embryos appear after 10 weeks.

Dehydration experiments:

Embryogenic cultures produced at CATIE were carried to UF by Dr. Dufour and Ms. Abdelnour. We collectively isolated somatic embryos and placed them in our dehydration apparatus at 70% relative humidity. This RH causes somatic embryos to dry to about 15-20% water content within one week. Embryos were separated into three different size classes, in order to test for the effect of developmental stage on embryogenesis. After two weeks of dehydration, embryos were rehydrated by placing them on germination medium. Control embryos of each stage were also placed directly on germination medium without dehydration. We found that 100% of the control embryos germinated, as evidenced by greening and enlargement, whereas none of the dehydrated embryos germinated.

We conducted a series of experiments that tested the effects of different humidities and pretreatments consisting of exposure to levels of ABA, BA, PEG and sucrose on somatic embryo survival after dehydration.

In an experiment comparing pretreatments of 0 and 5 μM BA with 3, 6, and 12% sucrose in all combinations, we found very little survival after 1 week of exposure to 70% RH. Only 3% of embryos pretreated with 12% sucrose and no BA survived (as evidenced by greening after rehydration).

An experiment comparing three embryo sizes (<2mm, 2-2.5mm, 2.5-4mm) dehydrated at 70 and 90% RH for 3 or 6 days was conducted. All embryos at 70% died, regardless of size or drying time. At 90% RH and 3 days of dehydration, 12, 36 and 40% of small, medium and large embryos survived, respectively. At 90% RH and 6 days of dehydration, no embryos survived. We felt that a critical parameter that influenced embryo survival had been determined. The difference between 3 and 6 days of dehydration was relatively small. Subsequent experiments were conducted at 90% RH and 6 days, with the expectation that pretreatments that increased survival could be determined in relatively little time.

A pretreatment utilizing 6% sucrose, 15 μM ABA and 5% PEG was compared to no pretreatment. Cultures were pretreated for 3 weeks before dehydration at 90% RH for 6 days. Both single embryos and embryo clumps were tested. Non-pretreated embryos and clumps did not survive. However, 7% of pretreated clumps and 18% of single embryos survived, as evidenced by greening, regrowth and resumption of secondary embryogenesis. Cultures that resume secondary embryogenesis are ideal for use in germplasm conservation schemes, since they are rapidly reproducing and can be used to supply clonal plants in a relatively short time period.

For coffee, several possible explanations for the lack of long-term dessication tolerance exist, including the possibility that the recalcitrance noted in nature is due to deelopmental mechanisims that cannot be overcome in vitro. However, in light of the positive results gained by others showing the effect of proper pretreatments on induction of dessication tolerance, further study is warrented. Overall, our results underscored the necessity of evaluating pretreatments that induce desiccation tolerance.

Cryopreservation experiments:

During this project, preliminaiy experiments were conducted to observe the effects of cryoprotectants, such as sucrose (up to 0.75 M), DMSO (5, 10 and 15%) and others, on germination of the embryos.

Three cryopreservation protocols initially were tested. The protocol reported by Bertrand-Desbrunais et al. (1988), which utilizes a culture pretreatment consisting of increasing concentrations of sucrose followed by infiltration with DMSO, was tested. Another protocol (Dereuddre et al., 1990) in which embryos are first placed in alginate beads, prior to dehydration in air was tested. Treatments included either rapid freezing by direct immersion in liquid nitrogen or slow freezing at either 0.5, 0.8 or 1°C/minute to -40°C prior to immersion in liquid nitrogen. Samples were either slow-thawed for 30 minutes or rapid-thawed in a 40°C water bath. Results from some of these experiments are given in Table 12, which shows that no pretreatment was equivalent to the DMSO pretreatment at week 4.

Table 12. Effect of pretreatments on survival of *Coffea* 'Robusta 3751' somatic embryos to cryopreservation in liquid nitrogen.

	%Survival	
Treatment	Week 3	Week 4
Isolated embryos	82	100
Pretreated w. 0.75M sucrose	79	93
Pretreat w. 5% DMSO	50	100
Slow-freezing before LN	0	80

Somatic embryos survived freezing, but did not germinate directly. Instead, they produce embryogenic calli and somatic embryos from which plants can ultimately be recovered. Robusta embryos showed the highest percent survival (71%).

Another protocol, developed at CATIE (Abdelnour-Esquivel et al., 1992) involved an air-dehydration pretreatment followed by rapid freezing in liquid nitrogen and then rapid thawing. Zygotic embryos were used here as a model for somatic embryos. This has not yet been successful and slow freezing will be tested in the future.

After the preliminary experiments described above, began using somatic embryos and concentrated on the freezing method described by Bertrand-Desbrunais *et al.* (1988). Somatic embryogenesis was achieved on coffee leaf discs, var. Robusta 3751 and Catimor (MP 547) on Yasuda medium. Bertrand-Desbrunais *et al.* utilized embryos derived from leaf calli of *C. arabica* var. Caturra Amarillo with the method reported by Dublin (1981). These authors used as a pretreatment, preculture of embryos at the globular stage for 24 hours in a standard medium containing 0.75 M sucrose. Embryos were then treated with 5% DMSO for 2 hrs. at 20°C, followed by freezing at a cooling rate of 5°C/min until -40°C was reached, at which point the somatic embryos were then plunged directly into liquid nitrogen.

At CATIE, we conducted variations of the above protocol, with the aim of: 1) improving the percentage of survival of frozen embryos and 2) simplifying the methodology by lowering the level of dehydration and accelerating the cooling pace.

We have been successful in cryopreserving somatic embryos of Robusta 3751 but not MP 547. Tables 12 and 13 illustrate the survival and regrowth percentages of Robusta somatic embryos after freezing in liquid nitrogen. Generally, the first signs of growth appear between the 5th and the 7th week after freezing. Table 12 shows three different replicated trials, all using the 0.75 M sucrose pretreatment for partial dehydration. It is obvious that there is a large difference between replications. The regrowth percentage at 15 weeks is 72% in one replication and 36% in the other two. Similar variability of results were also reported by Bertrand-Desbrunais *et al.* (1988), who reported that regrowth percentages at 17 weeks vary between 30 and 68%.

Due to this high degree of variation, it is currently advisable to incorporate many repetitions of each treatment in order to accurately determine differences in response between treatments. We found that the factors that affect success in cryopreservation, as measured by regrowth percentage, are: 1) somatic embryo quality (i.e. their ability to regenerate and germinate into plants), and 2) variations in the cooling process inherent to the device used. The best results are obtained with a pretreatment at 4°C for one hour with a cooling rate of 0.5°C/mn.

Another important difference in response between treatments is the speed of regrowth (Table 13). The pretreatment gave the most rapid regrowth. Controlled cooling to minus 15°C was the next best treatment. Direct plunge into LN was the worst treatment. These responses were observed for all replications.

Lack of success with MP 547 somatic embryos may be due to the inadequacy of both the somatic embryogenesis medium and the recuperation medium. During the process of somatic embryogenesis, many abnormal embryos are produced from this line and the germination percentage is very low. There is also some evidence of vitrification, a physiological disorder.

Several other trials are under evaluation. Due to poor results on Catimor, we re-evaluated embryogenic culture protocols. Several *C. arabica* genotypes were found to be responsive. For example, T-18135 (Icatu) produced embryogenic cultures on all media salt combinations tested, with 100% of explants producing embryogenic cultures and high numbers of somatic embryos on Yasuda and MS medium (Table 14).

Table 13. Percentage recovery of Robusta 3751 plants from somatic embryos (Test 4).

	Week						
Treatment	2	4	6	8	10	12	13
Pretreatment	57	86	86	86	86	86	86
Ctrl-15	38	62	85	100	100	100	100
Ctrl-20	0	63	75	88	100	100	100
Ctrl-30	0	2	20	90	90	90	90
LN	0	0	4	15	26	48	92

Table 14. Effect of macroelement formula and concentration on somatic embryogenesis of *'Coffea arabica* 'T-18135' (50 explants/treatment).

Macroelement treatment	Embryogenic cultures (%)	No. somatic embryos/culture
Yasuda's	100	12.1 ± 1.5
MS	100	8.2 ± 2.3
MS/2	77	3.1 ± 1.7
MS/4	71	3.6 ± 1.2
MS/10	68	2.5 ± 0.7
MS + 2 x KNO_3	100	6.3 ± 1.5

A final series of experiments evaluated the survival of embryos encapsulated in sodium alginate and cryopreserved.

Embryos were induced as previously described. Leaf sections from *in vitro* plantlets of Robusta were cultivated on a medium containing the M8 mineral salts, Morels vitamins, 1 mg/l of BAP and 7 g/l of agar. The pH was adjusted to 5.6 and the media autoclaved.

Embryos were isolated at globular stage and incubated on the media with increasing concentration of sucrose (0.1, 0.3, and 0.5 M) for 24 hours. Embryos were encapsulated in a sodium alginate at 3% and incubated at 4°C for 24 hours in a medium containing 1 M sucrose.

For desiccation the encapsulated embryos were placed on sterile filter paper in the laminar flow cabinet for a period of time (0.5 to 2.0 hours) and frozen rapidly (directly into liquid nitrogen) or slowly (using a programmable freezing machine) to 40°C prior to freezing in liquid nitrogen.

Embryos were planted in the recovery medium (described above) and evaluated for survival at 12-week intervals.

The survival of encapsulated embryos after cryopreservation treatments was evaluated. The effects of slow freezing and rapid freezing techniques discussed above are compared in Tables 15 and 16, respectively. The results suggest that rapid freezing results in a better survival percentage than slow freezing.

Measurement of weight loss of encapsulated embryos over time showed that weight dropped to 27.9 % of the initial fresh weight within two hours of exposure to air in a laminar flow hood (Table 17).

Table 15. Percentage survival of encapsulated embryos after rapid freezing in liquid nitrogen.

Desiccation duration (hours)	Embryo survival* (%)
0.5	7.0
1.0	14.2
1.5	11.7
2.0	60.0

*Average of 4 experiments

Table 16. Percentage survival of encapsulated embryos after slow freezing in liquid nitrogen.

Desiccation duration (hours)	Embryo survival* (%)
0.5	9.0
1.0	8.0
1.5	5.0
2.0	35.0

*Average of 5 experiments

Table 17. Humidity content of encapsulated embryos of Robusta using a laminar flow cabinet.

Desiccation duration (hours)	Fresh weight* (%)
0.5	75.4
1.0	86.8
1.5	39.8
2.0	27.9

*Average of 2 experiments with 3 replicates of 15 embryos each.

C) Impact, Relevance and Technology Transfer:

Results of the project cannot be used until more technical advancement occurs. This project set the ground work that, when fully realized, will have a significant impact on germplasm conservation of coffee and grape and will point the way for other crops as well. Both of these crops are currently difficult to conserve in clonal repositories due to maintenance costs. When obstacles related to genotype specific somatic embryo initiation and maturation/drying/quiescence are overcome, clonal germplasm of these crops will be stored more efficiently in a seed germplasm repository. However, technology development beyond the level reached by this project still is required.

Publications generated through this project had a stimulated similar research on somatic embryo maturation/drying/quiescence for other crops. This is illustrated by the rapid increase in number of publications devoted to aspects of synthetic seed technology that has occurred in the last few years (personal observation).

Technology transfer occurred through transfer of information and materials via mail and fax. There were two reciprocal visits by scientists during the projct. Gray visited CATIE in December of 1991. Dufour and Abdelnour visited UF in February of 1992. The visit to CATIE, which resulted in establishment of grape culture lines at CATIE and coffee culture lines at UF. Cryopreservation instrumentation at CATIE represents a significant technical advancement for that Institution. Transfer of dehydration protocols and training from UF to CATIE occurred during reciprocal visits. Overcomming institutional constraints was accomplished by the reciprocal visits.

D) Project Activities/Outputs:

Travel to two annual meetings each of the Society for In Vitro Biology and the American Horticultural Society were supported, in part by this project. However, travel funds were not a significant component of the budget.

Publications that arose from the project and that acknowledge USAID support:

Note: One copy of each publication resulting from this project was included with the required number of final report copies sent to the three mailing addresses (PO, MI & HCD).

1) A. Abdelnour-Esquivel, V. Villalobos, and F. Engelmann. 1992. Cryopreservation of zygotic embryos of *Coffea* spp. Cryo Letters 13:297-302.

2) M. E. Compton and D. J. Gray. 1993. Sucrose, abscisic acid and methyoglyoxal bis-(guanylhydrazone) affect grape somatic embryogenesis. Proc. Fla. State Hort. Soc. 106:124-128.

3) M. E. Compton and D. J. Gray. 1994. In vitro shoot micropropagation of 'Southern Home' muscadine grape. Proc. Fla. State Hort. Soc. 107:308-310.

4) D. J. Gray. 1992. Somatic embryogenesis and plant regeneration from immature zygotic embryos of muscadine grape (*Vitis rotundifolia*) cultivars. Amer. J. Bot. 79:542-546.

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6) D. J. Gray and M. E. Compton. 1993. Grape somatic embryo dormancy and quiescence: Potential of dehydrated synthetic seeds for germplasm conservation. In: K. Redenbaugh, Ed., Synseeds: Applications of synthetic seeds to crop improvement. CRC Press, Boca Raton, pp. 367-379.

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8) D. J. Gray and L. A. Hanger. 1993. Effect of ovule maturity on recovery of zygotic embryos and embryogenic cultures from cultivars of muscadine grape (*Vitis rotundifolia* Michx.). HortScience 28:227.

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E) Project Productivity:

As discussed above, the project was not able to accomplish the ultimate goal of developing dehydrated, quiescent somatic embryos of grape and coffee genotypes. However, we made significant advancements in the recovery of embryogenic cultures from new species and cultivars of grape and demonstrated a new culture system that enabled the first example of somatic embryogenesis in *Vitis rotundifolia*, a member of the subgenus Muscadinia, which is genetically-distinct from the true bunch grapes (Euvitis). In addition to culture developmnt, we investigated parameters designed to enhance embryo maturity and dessication tolerance. Although we were able to make improvements for both grape and coffee, we fell short of the above-mentioned ultimate goal.

F) Future Work: Our research shows both the promise and the difficulty of developing adequate culture systems of grape and coffee. Research on pretreatments to improve desiccation tolerance should be continued. At present, the laboratory at UF remains intensely interested in overcomming genetic blocks to embryogenesis an are actively pursuing this avenue of investigation. We hope to continue the maturation studies, but do not presently have a source of funds to conduct adequate experimentation.

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II.2 Somatic Embryogenesis and the Technology of Synthetic Seed*

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1 Introduction

Synthetic seeds are functionally defined as somatic embryos engineered to be of use in commercial plant production (Gray 1990b). The actual form of synthetic seed (i.e., presence or absence of a synthetic seed coat, whether they are hydrated or dehydrated, quiescent or not, etc.) may vary depending on the specific crop application.

The first reference to the potential use of somatic embryos for propagation is generally credited to Murashige (1978) and efforts to engineer them into synthetic seed have been ongoing ever since (e.g., Gray et al. 1984, 1992; Kitto and Janick 1985a,b,c; Redenbaugh et al. 1986, 1987a,b, 1988, 1991, 1994; Fujii et al. 1987a,b, 1989; Gray 1987a, 1990b; Gray and Mortensen 1987; Stuart et al. 1987; Carman 1989; Janick et al. 1989; Kim and Janick 1989a,b; McKersie et al. 1989; Redenbaugh 1990; Senaratna et al. 1990; Gray and Purohit 1991a,b; Parrott et al. 1991; Gray and Compton 1993; Senaratna 1992; Attree and Fowke 1993). However, basic developmental mechanisms that contribute to the desirability of seed, such as onset of quiescence and ability to withstand dehydration, are either missing in all but a few embryogenic systems or have been simply overlooked (Gray 1990b).

* This is an updated version of a previous review (Gray and Purohit 1991a). Partial support for this project was provided by the International Board for Plant Genetic Resources (IBPGR) and the Program in Science and Technology Cooperation, Office of the Science Advisor, US Agency for International Development under Grant No. DHR-5600-G-00-0057-00. Florida Agricultural Experiment Station Journal Series No. R-03398.

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Biotechnology in Agriculture and Forestry, Vol. 30
Somatic Embryogenesis and Synthetic Seed I (ed. by Y.P.S. Bajaj)
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2 Somatic and Zygotic Embryo Development

2.1 Developmental Morphology

Somatic and zygotic embryos share similar gross ontogenies, with both typically passing through globular, torpedo, and cotyledonary stages for dicots (e.g., Ammirato 1987; Gray and Mortensen 1987) and conifers (Becwar et al. 1989), or globular, scutellar, and coleoptilar stages for monocots (e.g., Conger et al. 1983; Gray and Conger 1985b,c). However, significant differences exist that currently limit the use of somatic embryos for propagation. Monoembryonic species that produce zygotic embryos with a discrete filiform suspensor (such as most seed-propagated crops) often produce clusters of somatic embryos from a mass of embryonic tissue termed a proembryonal cell complex (Haccius 1978). This basic change in developmental pattern is likely due to differences between the in vivo (seed) and in vitro environments since immature zygotic embryos dissected from seeds and cultured often develop abnormally (Norstog 1965; Norstog and Klein 1972). Somatic embryos growing from proembryonal complexes tend to develop asynchronously so that several stages are present in cultures at any given time. Such somatic embryos initiated at different times are subjected to changing nutrient regimes as medium becomes depleted than replenished between and during subcultures. This leads to relatively extreme differences in development, even among embryos from a single culture. With these variable and nonregulated environmental conditions, somatic embryos often bypass maturation altogether, becoming disorganized, forming new embryogenic cells and contributing to asynchrony (Conger et al. 1989). Among embryos that grow to a relatively mature stage, embryonic organs may develop at different rates. This leads to precocious germination, in which only a root or a shoot is typically formed but normal rapid germination and growth to a plant do not occur. Somatic embryos also often exhibit structural anomalies such as extra cotyledons and poorly developed apical meristems (Ammirato 1987). These problems appear to be due to culture conditions and not factors intrinsic to somatic embryos since immature zygotic embryos exhibit similar irregularities when removed from seed and allowed to develop in vitro (Norstog 1965). Lack of synchronous cultures that produce uniformly mature somatic embryos is a serious obstacle to propagation, since embryo production systems that approach the uniformity of commercial seed production will be required for many applications (Haccius 1978; Gray 1987c). Methods to remedy some of these problems by regulating embryo development were discussed previously (Gray and Purohit 1991a).

2.2 Functions of Nutritive and Seed Coat Tissues in Seeds

Tissues that enclose embryos in seed can be broadly distinguished as having either a nutritive or protective function. A major difference between somatic and zygotic embryos is that the former develop naked without these tissues. Beyond protection and nutrition, such tissues create barriers to regulate gas exchange

(Nikolaeva and Katkevich 1961). For example, oxygen availability, which is limited by these tissues, influences embryo respiration (Nikolaeva and Katkevich 1961; Nutbeam and Duffus 1978), regulates embryo development, and prevents precocious germination (Norstog and Klein 1972; Carman 1988). In contrast, in embryogenic cell culture, availability of oxygen and other gases depends on the subculture interval, whether or not the vessel is tightly sealed as well as gas usage and evolution by cultured tissues. Studies to regulate oxygen availability in wheat (*Triticum aestivum* L.) cell culture demonstrated that a low oxygen concentration promoted embryogenic callus formation and suppressed nonembryogenic callus (Carman 1989). Since immature zygotic embryos that are removed from seed often do not develop normally, it can be inferred that the presence of seed tissues is a contributing factor to normal embryo development. The effects of nutritive and seed coat tissues were previously considered in greater depth (Gray and Purohit 1991a).

2.3 Quiescence and Dormancy

Perhaps the most significant developmental difference between zygotic and somatic embryos is that somatic embryos lack a quiescent resting phase (Gray 1987b). Generally, the resting phase is categorized as either "quiescent" or "dormant" (Bewley and Black 1985). Quiescence is a resting phase that can be reversed solely by the addition of water. Dormancy is a form of quiescence that requires factors in addition to water, such as cold or heat treatments, for resumption of growth to occur.

2.3.1 Desiccation Tolerance

Dehydration is of particular importance in onset and regulation of the resting phase. The embryo (and seed) is able to withstand dehydration only during a specific phase of development, termed the desiccation-tolerant stage (Gray 1987b; Senaratna et al. 1987,1990; Koster and Leopold 1988; Neumann et al. 1989). Prior to dehydration, metabolism is oriented toward accumulation of storage compounds, whereas after rehydration, storage reserves are consumed for germination. Lack of such developmental phases in conventional embryogenic culture systems may explain typical poor germination of somatic embryos. Physiological studies show that dehydration can dramatically alter biosynthetic pathways in somatic embryos. For example, Saranga et al. (1992a,b) showed that treatments with various osmotica resulted in increased endogenous proline levels and increased proline was associated with improved desiccation tolerance. Similarly, treatment of white spruce [*Picea glauca* (Moench) Voss] somatic embryos with ABA and polyethylene glycol, the latter of which functioned as a nonpermeating osmoticum, increased that amount of triacylglycerol ninefold (Attree et al. 1992). The same treatments that increased triacylglycerol also induced desiccation tolerance.

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2.3.2 *Quiescence in Somatic Embryos*

Several studies with carrot (*Daucus carota* L.) suggested that a resting phase could be induced in somatic embryos by drying (Jones 1974; Nitzsche 1978; Kitto and Janick 1985c). However, actual plant recovery from dehydrated somatic embryos was first documented in orchardgrass (Gray and Conger 1985a; Gray et al. 1987). In that study, somatic embryos were exposed to 70% RH air and stored for up to 21 days at 23 °C. Under these conditions embryos dehydrated rapidly, became discolored, decreased in size, and their outer cell walls collapsed. Embryo water content dropped from 83 to 13% within 24 h and was maintained over the entire storage period. This water content is similar to that of seeds maintained at 70% RH and is adequate for maintenance of viability during prolonged seed storage. The embryos were rehydrated by placement on solidified medium, during which they swelled rapidly, regained their normal white coloration, and germinated. However, only well-developed (possessing a visible scutellum and coleoptile), white, opaque somatic embryos were responsive. Such somatic embryos were structurally mature and contained starch and lipid storage compounds (Gray and Conger 1985b). When these embryos were stored in a dehydrated state for 21 days, 4% germinated and produced plants after imbibition (Table 1). The occurrence of quiescence in somatic embryos was demonstrated by this study.

Grape somatic embryos were subsequently dehydrated in the method described for orchardgrass and quiescence was induced (Gray 1987b,d, 1989). During dehydration, grape somatic embryos underwent morphological changes similar to those of orchardgrass. Their water content equilibrated to 13% when stored at 70% RH and they resumed a normal appearance after rehydration. Genotypic differences in response were noted; those that produced relatively well-developed somatic embryos were most responsive. After 21 days of dehydrated storage, 34% of embryos from one grape genotype produced plants following imbibition (Table 2). This study demonstrated that higher germination percentages were possible. Grape is discussed further in the next section in relation to dormancy.

Table 1. Germination of dehydrated orchardgrass somatic embryos after storage at 23 °C for 0, 7, and 21 days[a]. (Gray et al. 1987)

Response[b]	Days of dehydrated storage		
	0	7	21
No germination	126/28[c]	333/74	396/88
Germination – no further growth	180/40	81/18	36/18
Germination – viable plants	144/32	36/8	18/4
Total	450/100	450/100	450/100

[a]Embryos were imbibed on solidified medium after test storage periods.
[b]Embryos that produced root hairs, roots, coleoptiles, and/or shoots but failed to develop further were scored as germinated – no further growth. Those that produced green leaves and continued to grow were considered to be viable.
[c]Number/percentage of total.

Table 2. Comparison of dehydration and benzyladenine (BA) for inducing germination in grape somatic embryos.[a] (Gray 1989)

Treatment	Percent germination response[b]			
	Hypocotyl	Root	Cotyledon	Shoot
Dehydration	77	68	65	34
0.5 μM BA	100	92	98	12
Control	76	88	36	0

[a] Well-developed embryos were either dehydrated for 21 days at 70% RH and 27 °C, placed directly on medium with BA, or placed on basal medium (control).
[b] Germination response was based upon either enlargement and greening of hypocotyls and cotyledons or emergence of roots or shoots.

Quiescence also has been observed in somatic embryos of corn (*Zea mays* L.) by Compton et al. (1992), who showed that plants could be obtained from previously dried embryos, as well as in soybean (*Glycine max* Merr.) (Parrott et al. 1988). For soybean, plant formation from somatic embryos typically was spread over a period of 9 months. However, after 2 weeks, when results from seven genotypes were pooled, plant regeneration averaged 60% from embryos that were dehydrated compared with 1% for embryos that were not dehydrated. This study differed from the preceding dehydration studies in that it included a maturation period of 28 days.

The longevity record for survival of dehydrated somatic embryos was reported for alfalfa (McKersie et al. 1989). When high quality embryos were selected and treated with ABA before drying, 60% produced plants after 1 year of dried storage (Senaratna et al. 1990). Ability of the alfalfa embryos to withstand dehydration was due, in part, to ABA pretreatments, which induced a stage of desiccation tolerance as described in the previous section (Senaratna et al. 1987, 1989a,b). Provision of desiccation tolerance to somatic embryos clearly demonstrates that they are capable of entering complex developmental pathways normally associated with those of seed embryos when proper environmental conditions are provided.

Recently, methodology to induce quiescence in somatic embryos of white spruce was reported by Attree et al. (1991,1992). A nonpermeating osmoticum, such as polyethylene glycol, was required to induce desiccation tolerance, in addition to ABA. Desiccation tolerance did not occur in the absence of ABA or with permeating osmotica, such as sucrose. Gradual drying to about 29% water, with a total storage time of 14 days, allowed up to 81% of the embryos to germinate into plants after imbibition. This represents a significant advancement in light of the useful applications of synthetic seed technology in conifer improvement (see later discussion).

2.3.3 Dormancy in Somatic Embryos

Embryogenic cultures of grape represent a good example of an in vitro dormancy system since somatic embryos become well developed but still germinate poorly.

Dormancy of grape seed can be alleviated by cold stratification, after which germination occurs (Flemion 1937). Somatic embryos of grape are similar to seed in that they germinate better following cold treatments (Gray and Mortensen 1987). This suggests that the somatic embryos also are dormant. Studies of ABA concentration in embryogenic cultures showed a rapid increase during embryo development, which reached a peak at maturation (Rajasekaran et al. 1982). Cold stratification of somatic embryos resulted in a rapid decrease in ABA. Exogenously supplied ABA inhibited somatic embryo germination (Gray 1989). Because ABA is implicated as a controlling factor of dormancy in many types of seeds (e.g., Walton 1980; Bewley and Black 1985; Thevenot et al. 1987), it is plausible to consider that ABA also functions in grape somatic embryos. In contrast to ABA, exogenously supplied gibberellin (GA_3) caused grape somatic embryos to germinate and the concentration of endogenous GA-like compounds increased during cold stratification (Pearce et al. 1987). This suggests a simple endogenous control of embryogenesis and germination whereby ABA inhibits precocious germination and thus promotes normal development while GA causes germination to occur. Other examples of dormancy in somatic embryos were previously discussed (Gray and Purohit 1991a,b).

3 Genetic Variation from Cell Culture

Somaclonal variation has been documented for a number of culture systems (Redenbaugh et al. 1988; Bajaj 1990). In instances where somaclonal variation is present, inability to control it would invalidate the basic rationale behind synthetic seed technology. Rather extreme examples of this phenomenon include sterility arising in plants regenerated from long-term embryogenic cultures of carrot (Sussex and Frei 1968) as well as albinism in somatic embryo-derived plants of bromegrass (*Bromus inermis* L.) (Gamborg et al. 1970). Certain species differ in tendency to yield culture-induced genetic changes than others. For example, embryogenic response can be intentionally intensified in orchardgrass by recurrent cycles of culture initiation and plant regeneration, and the increased embryogenic response appears to be heritable (Hanning and Conger 1986). Conversely, repeated attempts to select increased embryogenic response in grape have been unsuccessful (Gray unpubl.). Further, two other studies have found very little variation in regenerated plants of barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.) (Ogura et al. 1987; Luckett et al. 1989).

Recently, the genetic stability of somatic embryo-derived poinsettia plants (*Euphorbia pulcherrima* Willd. ex Klotzsch) was compared with that of the embryogenic cells from callus, Erlenmeyer flask, and bioreactor cultures from which they were regenerated (Geier et al. 1992). Whereas the progenitor embryogenic cell cultures became increasingly unstable over time, with nuclear DNA contents ranging from 2C to 32C, the regenerated plants exhibited high uniformity in both ploidy level and morphology. These results suggest that the potential to form somatic embryos becomes impaired in cells with genetic abnormalities. These studies strongly indicate that the process of embryogenesis,

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at least for some species, is remarkably stable and resistant to random genetic change.

Possibilities for suppressing variation, when present, include frequent initiation of new cultures (Krikorian 1982) as well as the use of short culture growth intervals (Evans and Gamborg 1982), both of which reportedly minimize instability. Producing somatic embryos directly from other embryos without an intervening callus stage was used to reduce variation in oil palm (*Elaeis guineensis* Jacq.) (Brackpool et al. 1986). Despite the obvious importance of managing genetic variation in synthetic seed, it may be premature to anticipate problems before suitable prototype systems are in place.

4 Structural Aspects of Synthetic Seed

Synthetic seed will consist of either a quiescent or nonquiescent somatic embryo with or without a protective encapsulation. The exact form of synthetic seed that is required will depend upon its specific applications. Naked, nonquiescent somatic embryos, germinated in soil plugs, could be used to propagate certain ornamental crops that are now laboriously micropropagated by tissue culture (Table 3). Manpower reduction achieved by producing plants by somatic embryogenesis, when compared to existing micropropagation, would confer a cost advantage. Dehydrated, quiescent somatic embryos without encapsulation would be useful for germplasm storage since they can be hand manipulated and carefully stored in protective containers. Cost of manipulating somatic embryos for germplasm storage would be similar to that of seed. Nonquiescent encapsulated embryos could be useful for crops that are first grown in greenhouses before transplanting to the field, such as carrot and celery (Fujii et al. 1987a). However, for mass propagation of field crops, a protective encapsulation will be necessary.

Somatic embryo encapsulations are analogous to the seed coat and endosperm of normal seed. Encapsulations may provide physical protection to the somatic embryo and carry nutrients, growth regulators, antibiotics, fungicides, etc. to assist in germination and plant survival (Kitto and Janick 1985b,c; Redenbaugh et al. 1986,1988; Janick et al. 1989). Encapsulated somatic embryos could conceivably be handled as seed using conventional planting equipment. Both hydrated and dry encapsulations have been envisioned.

Table 3. Types of synthetic seed and crop applications

Synthetic seed type	Relative development cost	Relative cost per seed	Example of crop application
Naked embryo			
Nonquiescent	Low	High	Ornamentals
Quiescent (dried)	Low	High	Germplasm conservation
Encapsulated embryo			
Nonquiescent	Medium	High	Vegetable transplants
Quiescent (dried)	High	Low	Agronomic and conifers

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Nonquiescent somatic embryos placed in a hydrated encapsulation may be cost effective for certain field crops that pass through a greenhouse transplant stage such as celery (*Apium graveolens* L.) (Redenbaugh et al. 1986). A hydrated encapsulation, termed as hydrogel, that could provide protection and allow convenient handling has been developed (Redenbaugh et al. 1986, 1987a,b).

4.1 Delivery Methods for Somatic Embryos

To date, most embryo-to-plant systems require several intermediate steps prior to successful plant establishment in the field. For example, Haydu and Vasil (1981) germinated napier grass (*Pennisetum purpureum* Schum.) somatic embryos in tissue cultures until they grew into plants 1–5 cm long. These small plants were then transferred to culture tubes to establish a more vigorous root system. The next step was potting the plantlets into soil-vermiculite in a growth chamber, then acclimating the plants to lower relative humidities. Finally, plants were transplanted in the greenhouse and/or the field.

For some species, plantlet formation is a very slow process. Celery somatic embryos required 5 weeks before forming roots and leaves (Dunstan et al. 1982). After adequate growth was obtained, the plants were transferred to soil in propagation boxes in the greenhouse, then to individual pots. Spiegel-Roy and Vardi (1984) developed a procedure for plant regeneration of citrus that required several transfers from solid and liquid media. After plants reached a certain size, they were cultured in tubes on paper bridges for further development prior to transfer to soil. Sixteen to 18 weeks were required before plants obtained from somatic embryos were growing in the greenhouse.

Extensive transfer steps were also required to establish plants from papaya (*Carica papaya* L.) somatic embryos (Litz and Conover 1982). Embryos were germinated and formed plants on White's medium supplemented with 0.1–2 mg/l NAA and 0.05–0.2 mg/l BAP. Plants were then moved to a soilless potting mix and hardened off under intermittent mist for 2–2.5 weeks. These few examples illustrate the laborious and time-consuming task before plants from somatic embryos could be transplanted in the field.

Several delivery methods for propagating somatic embryos directly from in vitro conditions to the field or greenhouse have been proposed. They include: (1) simultaneous dehydration of somatic embryos using a water-soluble resin and planting in a wafer or seed tape (Kitto and Janick 1985a,b); (2) dehydration, then planting dried somatic embryos with a conventional drill (Gray et al. 1987); (3) encapsulation of singulated somatic embryos in an alginate gel capsule, then planting using a conventional drill (Redenbaugh et al. 1984, 1986; Jeon et al. 1986); and (4) gel seeding somatic embryos with fluid drilling equipment (Drew 1979; Baker 1985; Schultheis et al. 1986a,b; 1990; Cantliffe et al. 1987).

4.1.1 Dehydration and Encapsulation in Polyethyleneoxide

Dehydration of somatic embryos has been investigated with the intent of providing storage capability, and the ability to plant as normal dry seeds (Gray

and Conger 1985a; Kitto and Janick 1985a,b,c; Gray 1987a,b; Gray et al. 1987). Kitto and Janick (1985a,b) were the first to successfully dehydrate encapsulated somatic embryos of carrot and have them survive upon rehydration. Unspecified numbers of carrot embryos from suspension were incorporated with polyethyleneoxide (Polyox), a water-soluble plastic resin, and dried to form wafers under sterile conditions in a laminar flow hood at ambient temperatures and humidities (Kitto and Janick 1985b). A constant weight was achieved after 6.5 h of drying. Three percent of the embryos survived encapsulation once rehydrated, although it was unclear in the report as to whether plants formed. Embryo survival decreased rapidly over drying time. No survival was obtained when embryos were dried to a constant weight and not coated; however, some embryos (fewer than 1%) survived after 32 h when they were coated with Polyox. Hardening treatments, including 12% sucrose, chilling at 4 °C, High inoculum density, and/or 1 μM ABA prior to desiccation reportedly increased embryo survival compared with the nontreated control (Kitto and Janick 1985c). The use of a Polyox seed coat seemed to damage the embryos during the coating and drying process as supported by low percentage survival data. Dehydration of somatic embryos in Polyox does not appear to be a practical seeding method, especially since coating somatic embryos singly was difficult and that no plants formed after the coating process.

Recently, ABA-treated somatic embryos of carrot were successfully dried in an alginate encapsulation. Up to 68% of the embryos germinated from capsules that had been dehydrated by 92% after 10 days of storage (Liu et al. 1992).

4.1.2 Dehydration Prior to Encapsulation

Dehydration of somatic embryos as a separate step prior to encapsulation with a coating material was previously proposed (Gray 1987b,c; Gray et al. 1987). Somatic embryos of orchardgrass became quiescent when dried from 75 to 13% water (Gray et al. 1987). Developmental stage before desiccation was critical for embryo germination and plant formation. Only those embryos with well-differentiated scutellar and coleoptilar regions prior to desiccation were able to germinate and/or form plants after imbibition (see Sect. 2.3.1 for more details on dehydration).

4.1.3 Encapsulation in Soft Gel Capsules

A third delivery method proposed was encapsulation of somatic embryos in a soft gel capsule (Redenbaugh et al. 1984, 1986; Jeon et al. 1986). Several compounds were tested for suitability for encapsulation, but calcium alginate, a food thickener derived from brown algae, was selected due to its low embryo toxicity and ease in forming capsules around the somatic embryo (Redenbaugh et al. 1986).

Gel capsules can be amended with nutrients, plant growth regulators, and carbohydrates to facilitate the rapid growth and survival of embryos. Most

encapsulation research has dealt with alfalfa somatic embryos, which have consistently averaged 60% plant formation under in vitro conditions and 20% when grown in the greenhouse (Fujii et al. 1987a,b). The best plant recovery frequency was 65% with celery somatic embryos when grown under in vitro conditions utilizing carefully selected embryos.

Examples of other crops where encapsulated somatic embryos were shown to form plants in vitro were *Brassica* species, carrot, cotton, lettuce, and corn (Redenbaugh et al. 1987a). Although encapsulated somatic embryos have been successfully converted to plants in several species, there are problems with the alginate capsules. Water-soluble nutrients have been reported to rapidly leach out of the capsule (Redenbaugh et al. 1987b). Respiration has been shown to be reduced in encapsulated seeds due to poor gas exchange (Redenbaugh et al. 1993). Root and shoot emergence have also been inhibited. In addition, Redenbaugh et al. (1987b) states that there is "lack of automation technology to allow medium to large-scale production of single-embryo capsules." Finally, storage may be a problem since embryo viability may decline over time due to inhibition of embryo respiration in the capsule (Fujii et al. 1987a,b). This seeding technology is relatively new and will require much more intensive study before it can be used.

4.1.4 Fluid Drilling

Fluid drilling is the sowing of seeds in a protective flowable gel. The system was developed in England during the 1970s (Gray 1981). The concept of fluid drilling zygotic seed was first conceived by Currah et al. (1974), while Drew (1979) was one of the first to suggest the idea of fluid drilling somatic embryos.

Actively growing or pregerminated embryos can be sown in the gel without damage. The gel can also be amended with nutrients, carbohydrates, beneficial microorganisms (mycorhizae, bacteria), and pesticides to stimulate embryo growth and improve plant survival. The microenvironment created around the seeds when fluid drilled can be modified and optimized such that the above advantages can be realized (see Chap. II.5, this Vol.).

4.1.5 Gel Type

Due to their various chemical compositions, certain gels may be better carriers for fluid drilling. For example, some gels may facilitate better oxygen movement, some may contain nutrients which enhance plant growth, and others may hold more moisture. Several gels have been used for drilling zygotic seed. Some of the more commonly used gels are: Laponite (magnesium silicate clay), Liqua-gel (potassium starch acrylamide), Planta-gel (copolymer of potassium acrylate and acrylamide), Terrasorb (starch or synthetic copolymer), Hydrozorb 30 (potassium acrylate), and N-gel (various cellulose-based materials, formerly called Natrosol).

A laboratory study by Brocklehurst (1979) determined that the polyacrylate mineral colloid gel was superior to the natural gum gels since the latter caused

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lettuce seedlings to senesce 4 days after planting. Sosa-Coronel and Motes (1979) evaluated seven commercial gels for planting carrot, onion, and pepper seed. Pregerminated seeds were suspended in gels for 4 and 24 h prior to seeding in the greenhouse. Superior seedling growth was obtained for all species sown with Viterra 2, Natrosol 250, and Bacto-Agar gels whereas Gum Blend and CLD were toxic.

Oxygen movement is dependent on gel type and temperature. Frazier et al. (1982) determined that oxygen movement in gels was slower as temperature decreased. Slower oxygen movement in the gel could be critical since low temperatures at planting might restrict the metabolism of the pregerminated seed. Seed viability was related to oxygen diffusion in the gel. Of all the gels tested, Natrosol had the highest oxygen diffusion and led to the most radicle emergence and growth of snapdragon seedlings when suspended in gel. Pill and Fieldhouse (1982) ran a similar comparison and also found that Natrosol was the only gel that tomato seed could be stored in for more than 4 days without reducing the percentage and rate of emergence. Bryan et al. (1982) observed slower plant emergence and reduced pepper stands when germinated seeds were sown in Laponite gel than without gel under wet growing conditions. Laponite has been reported to reduce O_2 uptake by the seed (Brocklehurst 1979).

4.1.6 Protection from Damage and Desiccation

Gels will protect actively growing seeds with or without roots. In addition, gels absorb water to many times their weight. This keeps moisture around the seeds and reduces the possibility of desiccation. Gels may retain moisture around the seeds several days after planting (Bryan et al. 1978).

4.1.7 Gel Additives

Ghate (1982) studied the rheological properties of gels when amended with chemical additives. Little change occurred in the gel without additives after 1 day when temperatures varied between 10 and 37 °C. The addition of fertilizer caused gels to become thin, while relative consistency was maintained with the addition of more gel.

Nutrient salts have been consistently added to gels at planting to enhance germination and early seedling growth from zygotic embryos. Nitrogen, phosphorus, and potassium were added to guar gum gel with little effect on lettuce or cabbage growth (Costigan and Locascio 1982). Finch-Savage and Cox (1982) reported improved early seedling growth of carrots with the addition of monosodium phosphate at concentrations up to 30 g/l, but nitrate (5–20g/l) reduced seedling growth. In a similar study, Finch-Savage and Cox (1983) obtained earlier maturity in lettuce and onion when pregerminated seeds were fluid drilled in gel supplemented with either sodium phosphate or ammonium nitrate compared with nontreated, dry sown seed. No benefit was obtained when less than 10 g/l ammonium nitrate was incorporated in the gel.

Faster, more complete germination of pepper seeds was reported when cytokinins, humic acids, or alpha-keto acids were added to the gel and planted under wet environmental conditions (Bryan et al. 1982). The addition of GA_3 and $GA_{4/7}$ to gel at 2 and 2.5 mg/l increased the growth of tomato seedlings compared with gel without GA (Ohep and Cantliffe 1980). Tomato plants were taller when the gel was supplemented with either Cytex or Cytozyme, while increased plant height, dry weight, and leaf area under field conditions were obtained when humic acids were incorporated. GA and diphenamide hastened the emergence rates of pepper by 1 to 3 days (Ghate and Phatak 1983).

Various pesticides have improved plant establishment. Ohep et al. (1984) reduced damping off of tomato seedlings by incorporating ethazol plus thiophanatemethyl, fenaminosulf, and ethazol alone or combined with benomyl, chloroneb, or captan into the gel. White (1979) controlled white rot of onions more efficiently by placing 25 mg active ingredient/l metalaxyl in the gel, whereas much higher concentrations of metalaxyl were required to overcome the problem when incorporated in the soil. Similarly, iprodione, when placed in gel, effectively controlled white rot of salad onion at one-quarter the rate used for dry seeds (Entwistle 1979).

Hadar et al. (1984) added the biocontrol agents *Trichoderma harsianum* and *T. kininoii* to gel to prevent seed rot in peas and navy beans when sown in *Pythium*-infested soil. They determined that 95% of the plants emerged when seeded in gel amended with *T. harsianum* and *T. kininoii* compared with 15% when planted with dry seeds. Inclusion of *Rhizobium* in the gel resulted in more than three times more root nodules on navy bean (*Phaseolus vulgaris* L.) compared to inoculation of dry seed (Hardwick and Hardaker 1977).

The growth of *Trichoderma hamatum*, a biocontrol and antagonist used to control *Rhizoctonia solani*, was compared in Planta-gel, Natrosol, and Laponite gel carriers (Mihuta-Grimm and Rowe 1986). Superior growth of *Trichoderma* isolates was obtained in Natrosol versus Planta-gel and Laponite gels. Due to better growth of the antagonist in Natrosol, this gel was supplemented with *Trichoderma* to control damping off when fluid sowing radish seeds. Four delivery systems for application of *Trichoderma* isolates were compared: (1) fluid drilling in Natrosol; (2) coated seed; (3) application of wheat-bran grown isolated applied in the furrow before planting; and (4) soil drenching with 5-day broth-grown cultures. The best disease control was consistently obtained with fluid drilling.

4.1.8 Gel Studies with Somatic Embryos

Baker (1985) determined that a mixture of peat and vermiculite was superior to vermiculite alone as a soil planting medium for carrot somatic embryos since the former alleviated water stress. However, vermiculite was the best soil cover since it did not crust and inhibit emergence. Baker also compared Natrosol and Planta-gel for suitability as carriers for fluid drilling somatic embryos. No difference was measured between gels with respect to embryo emergence, which

was approximately 5 to 30% under in vitro conditions. The polyacrylamide material was used in subsequent greenhouse studies.

Schultheis et al. (1986a,b,1990) reported that somatic embryos of sweet potato grew quite rapidly in N-gel when compared to three other gel carriers. All embryos suspended in the gel were viable after 6 days and up to 62% of the embryos formed plants. Both NAA and BAP improved plantlet formation in the gel and a sucrose concentration of 1.6% resulted in the highest plantlet formation. Up to 25% more plants were obtained when elongated, torpedo-stage (expanded mature) embryos were sown in the gel carrier compared with embryos sown at the torpedo (immature) or cotyledonary (mature) stages.

Although somatic embryos are similar to zygotic embryos, the former lack many of the stored reserves zygotic embryos have, available for their early seedling growth. Thus, it is important to determine which "growth factors" are necessary to achieve rapid and complete plant formation from somatic embryos. It is also necessary to use a synthetic seeding method which could be easily adapted for field use. The incorporation of additives around the embryos to sustain and improve their growth and development into plants would be required for the successful implementation of synthetic seeding.

5 Automation of Synthetic Seed Production

The commercial application of somatic embryogenesis for many crops requires high volume, low cost production, which invariably translates to a requirement for automated production and harvest. Relative need for automation will vary depending on crop application. Synthetic seed production can be separated into several discrete steps, each of which are amenable to automation: (1) production of cell cultures and somatic embryos; (2) sorting and harvesting of somatic embryos; and (3) encapsulation and dehydration.

5.1 Automation of Cell Culture and Somatic Embryo Production

Production unit operations vary considerably depending on the culture system used. While mass production of somatic embryos is probably best accomplished in liquid medium, which can be scaled up in bioreactors (Redenbaugh et al. 1988), a number of possible alternatives have been explored. For Petri plate-based protocols, production involves subculturing callus from plate to plate under aseptic conditions. Subculturing involves medium preparation, plate lid removal, harvesting calli, sieving and dispensing calli on new plates. Kurata and Futaya (1992) developed an automatic sieving system applicable to many of these operations. The goal of this system was to select appropriately sized calli for the production of carrot somatic embryos. However, the system was not as effective as manual sieving, but did demonstrate the validity of the concept.

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For a liquid-based production protocol (primarily bioreactors), unit operations are typical of chemical process control tasks. Durzan and Durzan (1991) list the process control objectives as: (1) suppression of the influence of external disturbances on the somatic embryogenesis process; (2) assurance of the stability of embryo development; and (3) optimization of the overall, long-term performance of somatic cells and embryos. Process control requires identifying the outputs to be controlled (e.g., embryo production rate and maturity), how these outputs are to be quantified, and the inputs to be used to manipulate the outputs. In some instances production objectives may be met by controlling elemental physical and chemical parameters such as temperature and pO_2 without the need to measure the output variables. A successful example of this type of process control is the model system for the bioreactor production of poinsettia globular somatic embryos (Preil 1991). The manipulated variables (inputs) for this system included temperature, agitation, pH, pO_2, redox potential, and pCO_2. The controlled variable (output) was embryo production rate. Relationships between the controlled and manipulated variables were established by off-line experimentation which avoided the need for on-line monitoring of embryo production.

On-line monitoring of the callus production phase of the somatic embryogenesis process was demonstrated by Harrell et al. (1991). A machine vision system was used to nondestructively monitor the growth of sweet potato callus during a 10-day culture period in an airlift bioreactor. Growth data obtained with the system included overall reactor population and population estimates for the 200–1200 μm fractions at 200-μm intervals. A model of callus growth was developed to explain the mechanics of callus enlargement. The model was based on the assumptions that (1) the calli could not shrink or subdivide, (2) there was a fixed percentage of the initial population within each fraction that was nonviable, and (3) growth rates did not vary with time during the culture period. It was determined with this system that growth rates and nonviable ratios decreased as fraction size increased.

Obstacles to automated production lie primarily in the biological systems. While well-developed somatic embryos capable of surviving direct field planting have been produced in liquid flask culture (e.g., Gray et al. 1992), there have been difficulties involved with scale-up of cultures in larger bioreactors. The main obstacle is that somatic embryos generally do not develop as well in liquid culture medium, when compared to solid medium. For example, plant recovery from suspension culture-derived somatic embryos of alfalfa was less than from callus and decreased still more when scale-up to a larger bioreactor was attempted (Stuart et al. 1987). Improvements in bioreactor design are needed as well as a better understanding of somatic embryo development under liquid culture conditions to achieve progress in large-scale production.

5.2 Automation of Somatic Embryo Selection and Harvest

Separation of viable embryos from other tissue is necessary due to lack of synchronization of embryo maturity and the proliferation of aberrant structures

in many systems. Machine vision has been the primary technology used to sense viable embryos (see Chapter I.7, this Vol.). Robotic and fluidic approaches have been developed to do the physical sorting. Postharvest encapsulation has been developed to help improve the embryo's chance of survival and germination.

Sakamoto et al. (1992) developed a system that automatically encapsulated somatic embryos in gel beads, which were optically sorted, and then sowed in cells of a conventional growing tray. The self-breaking gel beads were composed of modified hydrogel as described above and contained a sucrose sustained-release microcapsule. The embryos were approximately 5 mm in length at the time of encapsulation and were subjected to a pre-encapsulation treatment process, which included mild dehydration (Onishi et al. 1992). Approximately one-third of the beads produced by the system contained no embryo, one-third contained a single embryo and one-third contained multiple embryos. Optical sorting was implemented to collect only those beads whose green content was higher than an established threshold. This eliminated empty beads and those which contained pale green embryos. The system was capable of producing and sowing 70 000 synthetic seeds per day and test results showed a plant recovery rate of 52%.

Manual sorting of somatic embryos prior to germination is often performed in an attempt to enhance the plant recovery rate. Visual cues predominate in this process and several researchers have attempted to replicate and improve upon human sorting using machine vision. The early work by Grand d'Esnon et al. (1988) demonstrated the technical practicality of recognizing torpedo-shaped sweet potato embryos with machine vision. Cazzulino et al. (1991) extended the concept and quantified embryo production experiments with machine vision by counting the occurrence of globular, heart-, and torpedo-shaped embryos in carrot suspension cultures. Kurata et al. (1991) developed a thinning algorithm to recognize torpedo-shaped carrot embryos. In this approach, the outline of an embryo was degenerated to a skeleton figure, which facilitated detection and analysis of cotyledons. The skeleton approach was also employed by Cheng and Ling (1992) to analyze images of coffee somatic embryos. The algorithm was 74% successful at distinguishing between 69 torpedo and nontorpedo stage embryos judged by a human expert. Among misclassifications, 13 human-judged torpedo embryos were classified as nontorpedo stage embryos and five nontorpedo stage embryos were incorrectly classified as torpedo embryos.

Kurata and Shono (1992) developed a recognition algorithm based on Fourier coefficients extracted from embryo outlines. Under limited testing, this algorithm coincided with human judgment at the 74% level. Birch and Norway spruce somatic embryos were classified with a machine vision system by Hämäläinen et al. (1992b, 1993). The classification algorithms were based on generic size and shape features in addition to morphological specific features, which quantified certain cotyledon characteristics. When tested on an independent set of birch somatic embryos (a set that excludes any images used to develop the classification algorithm), only 0.1% of nonembryo objects were classified as embryos and only 17% of the good embryos were discarded by the classifier.

A novel approach to image classification of somatic embryos was described by Moltó and Harrell (1992). This classification algorithm utilized a neural

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network to emulate the judgment of a human experienced in selecting viable embryos. The network processed 17 geometric features extracted from an embryo's outline. The feature set included size measures such as embryo length as well as shape measures such as circularity and roughness. Alternatively, the neural network could process 32 radii lengths from the perimeter centroid to 32 perimeter points at a constant angular increment of $2\pi/32$ radians. Angular intervals were taken counterclockwise starting from the principal axis of the embryo. On an independent test set, the neural network classifier correctly selected 75% of the embryos deemed desirable by the human expert and correctly rejected 74% of the malformed, immature, or nonembryo structures.

Hämäläinen et al. (1992a) discussed a prototype system that could mechanically harvest somatic embryos targeted by the machine vision classifier using a robotic manipulator. A fluidic, in vitro approach to embryo monitoring and sorting was developed for liquid-based, bioreactor culture systems by Hood (1992). A vision system was used to rate the viability of embryos as they were pumped through a 3-mm, square glass conduit. Embryos were ranked with the neural network described in Molto and Harrell (1992). Embryos targeted by the network were ejected from a gap in the flow conduit by a precisely timed injection of culture medium from a control nozzle. The positions and velocities of embryos between the imaged section of the conduit and the harvest gap were monitored by an object tracker employing 30 LED/photo-diode pairs mounted along the conduit. Objects not harvested were routed back into the reactor. The harvester was designed to sort one embryo per second. In nonaseptic tests 28% of the desirable embryos from a test population were harvested improving the homogeneity of the harvested population from 56 to 88% (Harrell et al. 1992).

5.3 Automation of Encapsulation and Dehydration

It may be possible to combine the steps of embryo dehydration and encapsulation in an automated process. Dehydration and encapsulation may be accomplished simultaneously by using an osmotically active synthetic seed coat (Gray 1987b). Hydrated embryos placed in such a material would begin to lose water during the capsule hardening process. Final water content could then be controlled by composition of the encapsulation.

6 Estimated Cost of Synthetic Seed

The overall expense of introducing synthetic seed technology for a given crop will result from a combination of development and seed production costs and will vary depending on the level of sophistication required for a given application (Table 3). For example, use of naked, nonquiescent embryos will involve less preliminary development expense but the cost of manipulating each embryo to a plant will be relatively high. In contrast, development of automated culture and

encapsulation systems will be relatively expensive but the mass production of synthetic seed with convenient storage and handling characteristics will result in relatively low plant production costs. It is impossible to determine actual costs for all types of synthetic seeds at this early stage of development since several key components of technology (i.e., automated culture systems and hardened encapsulations as described above) have not yet been refined. However, use of hydrated encapsulated alfalfa somatic embryos, which involves much hand manipulation to produce, would result in a cost of US $0.033 for each greenhouse transplant (Redenbaugh et al. 1987a). This level of synthetic seed technology would be effective if adapted to certain crops with high per-plant value as discussed below. Further, cost per plant would undoubtedly be greatly reduced with an automated system.

7 Crop Applications for Synthetic Seed

Potential applications of synthetic seed will vary from crop to crop depending on the relative sophistication of existing production systems and the opportunities for improvement. Whether or not a cost advantage results from synthetic seed will ultimately determine its commercial use. As discussed above, for seed-propagated agronomic crops, relatively sophisticated quiescent, encapsulated somatic embryos produced en masse will be necessary in order to achieve adequate planting efficiencies. However, for vegetatively propagated crops, particularly those with a high per-plant value, naked, hand-manipulated, nonquiescent embryos may be cost effective. A discussion of potential applications of synthetic seed technology for several specific crop types follows.

7.1 Ornamental Crops

Ornamental crops have a yearly value of over $ 2.4 billion in the USA (Anonymous 1990). Many are laboriously micropropagated via tissue and organ culture where per-plant production costs can exceed $ 0.50 (Florkowski et al. 1988). Most of this cost is due to manpower needed for multiple culture and rooting steps. Substitution of embryogenic culture systems for such crops would greatly reduce labor costs since somatic embryos could be mass produced from callus, then hand selected and placed directly into planting flats, resulting in rooted plants. This would eliminate several labor-intensive steps. The implementation of synthetic seed for ornamental crops is compelling since a relatively modest level of technological development would result in lowering existing costs. Somatic embryogenesis has been reported for a number of ornamental species (Gray and Purohit 1991a). Additional ornamental systems surely will be developed and refined as pressure to decrease labor costs intensifies.

7.2 Vegetable Crops

Certain vegetable crops are also early candidates for implementation of synthetic seed technology due to high seed and/or propagation costs in conjunction with high per-plant value. Generally, hybrid varieties bring higher prices. Asparagus (*Asparagus officinalis altilis* L.) and certain cucurbits have seed costs that approximate those estimated for encapsulated, nonquiescent somatic embryos (i.e., > $ 0.033). For seedless watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai), seed cost can be so high (up to $ 0.35 per seed) as to approach that of tissue culture micropropagation. Considering that often only 30% of seedless watermelon seeds germinate, the cost of producing a single seedling can be as high as $ 1.05. For this crop, synthetic seed could reduce per-plant costs by circumventing barriers to seed production. However, seed costs are volatile and the first report of somatic embryogenesis has just occurred for watermelon (Compton and Gray 1993). Therefore, although the application is compelling, much research and development would be necessary to commercialize synthetic seed technology for this crop. For sweet potato, 1 acre of nursery space is consumed to produce enough plants for 10 acres of production field (Cantliffe et al. 1987). In this instance, implementation of synthetic seed would dramatically lower production costs by eliminating nursery requirements.

7.3 Conifers

Forest conifers can be propagated economically only by seed. Improvement via conventional breeding is extremely time-consuming due to the long conifer life cycle. Furthermore, conifers are highly heterozygous so that seed from outstanding individuals does not necessarily result in improved progeny. Superior breeding stock has been developed, but there is a lag time to produce improved seed in an orchard setting. Synthetic seed offers the possibility of cloning outstanding elite trees at reasonable costs, thus circumventing years of development (Farnum et al. 1983). To date, well-developed somatic embryos have been obtained for a number of conifer tree species (e.g., Gupta and Durzan 1987; Becwar et al. 1989; Attree et al. 1991).

7.4 Forage Crops

Synthetic cultivars of seed-propagated, self-incompatible crops such as alfalfa and orchardgrass are developed laboriously by selecting phenotypically uniform but genetically distinct lines (Gray et al. 1987, 1992; McKersie et al. 1989; Gray 1990a). These lines are then allowed to cross-pollinate for seed production. Such seed is nonuniform and each resulting plant is a potentially distinct genotype. The nature of this breeding system makes it difficult to incorporate specific new genes into existing lines. Use of synthetic seed would allow single, outstanding hybrids to be utilized as cultivars since self-fertilization would not be needed for seed increase. Such cultivars would be genetically uniform. Although excellent

embryogenic culture systems exist for both alfalfa (e.g., Walker and Sato 1981) and orchardgrass (e.g., Gray et al. 1984), a limitation to this application of synthetic seed technology is the low per-plant value and the low cost of existing seed. An intermediate use of synthetic seed for these crops may be to increase parental lines prior to establishment in open crossing blocks (McKersie et al. 1989; Gray 1990a; Gray et al. 1992).

7.5 Fruit and Nut Crops

Planting efficiency of crops that are currently vegetatively propagated due to self-incompatibilities and long breeding cycles, such as fruits and nuts etc., could theoretically be increased by using synthetic seed instead of cuttings. But, since existing methods tend to be cost effective, developmental costs of synthetic seed would not likely be justified. Direct planting via synthetic seed is further complicated for varieties that require grafting to a rootstock. Use of synthetic seed for germplasm conservation of these crops could be highly advantageous, however. For example, germplasm of unique clones maintained as living plants in field gene banks (Towill 1988). This method of conservation is expensive and subject to loss from environmental disasters. The use of in vitro conservation methods for these crops, in addition to recalcitrant-seeded species, has been emphasized (Bajaj 1986; Withers 1989). Use of synthetic seed would allow clonal germplasm of grape to be conserved in seed repositories. More genotypes could be conserved since space problems would be eliminated. Moreover, development costs would be reduced since automated production equipment for mass production would not be needed for the relatively small number of synthetic seed required. This method of germplasm conservation would be particularly useful for tropical species where existing conservation is inadequate or nonexistent. Grape is a good experimental prospect since well-developed somatic embryos have been obtained (Gray 1987b,1989; Gray and Mortensen 1987). The prospects of using synthetic seed technology for grape germplasm conservation was previously discussed in depth (Gray and Compton 1992).

7.6 Cotton and Soybean

Commercial quantities of hybrid seed are difficult to produce for certain seed-propagated crops such as cotton (*Gossypium hirsutum* L.) and soybean due to cleistogamous flowers and/or problems with flower abscision. Thus, seed of most existing cultivars is derived from self-pollination. However, relatively small numbers of hybrids can be produced laboriously by extensive hand pollination and subsequently mass produced by use of synthetic seed. Hybrid vigor could then be exploited at the production level. Somatic embryogenesis has been obtained for both cotton (Finer 1988) and soybean (Ranch et al. 1985), although the commercial potential of synthetic seed for such crops is unclear at this time, considering the moderate cost of existing seed and the possibility of developing chemical emasculants to produce male-sterile plants in the field.

7.7 Hybrid Cereals

Synthetic seed technology provides the possibility of circumventing the need for inbred and male-sterile parental lines in hybrid seed production. For example, the hybrid corn industry relies on inbred parentals to produce uniform hybrid seed. Mass hybridization is possible by using male-sterile lines as females. Increased production costs over open-pollinated seed that are incurred by use of inbred and male-sterile lines are more than offset by the resulting yield and quality conferred by hybrid vigor. However, development and maintenance of these parental lines consume much of the time and resources of a breeding program and integration of new germplasm is slow. An intriguing possibility is the use of synthetic seed to propagate new hybrids and eliminate the need for parental inbreds and male steriles altogether. This would facilitate commercialization of new hybrids and would probably stimulate competition since newcomers could produce cultivars without an existing stock of parental inbreds. Although somatic embryogenesis of corn is well described (Kamo et al. 1985), it is unclear whether the successful hybrid corn industry would accept this concept.

8 Conclusion

The structural similarities between somatic embryos and zygotic embryos are the basis for interest in synthetic seed technology. However, functionally, somatic embryos have yet to match the convenience of seed. A number of obstacles, for example, problems with mass production, encapsulation, and uniformity, must be overcome before synthetic seed can be useful for most crops. Many of these problems are probably related to shortcomings of contemporary in vitro culture systems. In vitro culture presents a very different environment compared to that in which zygotic embryos develop (i.e., seeds). The environment of the developing seed is dynamic and complex, probably causing many functional attributes of zygotic embryos that we would desire to have expressed in somatic embryos.

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Cryo-Letters 13, 297-302(1992),
published by Cryo-Letters, 7, Wootton Way, Cambridge CB3 9LX, U.K.

CRYOPRESERVATION OF ZYGOTIC EMBRYOS OF *COFFEA* SPP.

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SUMMARY: Zygotic embryos of *C. arabica*, *C. canephora* and the hybrid arabusta at various maturity stages were successfully frozen in liquid nitrogen after partial dehydration in the air current of a laminar flow cabinet followed by rapid freezing. The optimal desiccation period was 0.5 hour. Survival was greater with embryos at an intermediate stage of maturity and ranged from 41.6% (28.9% water content) for *C. canephora* to 95.8% (15.8% water content) for *C. arabica*.

KEY WORDS: *Coffea*, zygotic embryos, cryopreservation, desiccation, germplasm storage, plant recovery.

INTRODUCTION

Seeds are generally used for germplasm storage. Seeds that resist high levels of desiccation and low temperatures without suffering damage (orthodox seeds) can be stored for long periods in seed genebanks with practical and economical results (1). However, there are seeds which cannot whithstand these storage conditions without rapidly loosing viability or being killed. These are the so called recalcitrant seeds (2, 3).

Coffee seeds have been considered recalcitrant based on their storage behaviour (4, 5, 6, 7). However, results from several studies showed that they did not behave as truly recalcitrant, since they resisted to relatively high levels of desiccation but viability loss increased with reduction in moisture level or temperature (8, 9, 10, 11, 12). Ellis and coworkers (8) suggested a new category of seed storage behaviour intermediate between orthodox and recalcitrant, which would include those seeds that as coffee can be dried but are injured by low temperature.

High germination rates could be maintained for 1 or even 2 years when coffee seeds were stored at about 10% moisture content and 15°C (8, 11), but no effective procedure has been described for long-term storage of these seeds.

Cryopreservation (liquid nitrogen, -196°C) has been shown as a feasible method for long-term storage of germplasm. Once the material is frozen all metabolic

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functions cease and no cell deterioration or mutation occurs, therefore genetic stabilitity of the stored material is maintained (13). This technique is specially useful for the long-term preservation of all those troublesome species which cannot be maintained under conventional storage methods.

For coffee germplasm preservation, cryopreservation can be useful as an alternative to field collections providing safe and economic long-term storage. Some preliminary attempts to cryopreserve coffee have been performed. Becwar and co-workers (7) tried to cryopreserve seeds of *C. arabica*. The results showed that even though seeds tolerated dehydration to as low as 8% moisture content, they did not withstand freezing in liquid nitrogen. In addition, successful cryopreservation of somatic embryos was reported for *C. arabica* (13) and *C. canephora* (14). The embryos were treated with high sucrose concentrations and slow freezing to -40°C prior to immersion in liquid nitrogen.

In the present study, the ability of zygotic embryos of coffee to withstand freezing in liquid nitrogen was examined. Embryos isolated from fruits at three different maturity stages were successfully cryopreserved following a dehydration pretreatment.

MATERIALS AND METHODS

Zygotic embryos of *C.arabica* cv Caturra, *C. canephora* and the hybrid arabusta (*C. arabica* x *C. robusta*) were used. They were extracted from fruits at three different maturity stages, based on skin coloration: green fruits, collected 2 months before harvest, yellow fruits, collected about 4 days before they turned red and red fruits ready to harvest. The fruits were disinfected by washing them with tap water and soap, 70% ethanol for 15 min and rinsing 3 times with sterile water. Embryos were isolated under aseptic conditions. They were cultivated on a medium containing the mineral solution and the vitamins of Murashige & Skoog (15), 30 $g.l^{-1}$ sucrose, 1 $mg.l^{-1}$ benzyladenine and 2 $g.l^{-1}$ gelrite. The pH was adjusted to 5.7 before autoclaving. Gibberellic acid (GA_3) at various concentrations was added in the recovery medium during some experiments.

For cryopreservation, the embryos were placed on small pieces of filter paper and dehydrated under the sterile air of a laminar flow cabinet for increasing lengths of time (0 to 2 hours). At the end of each dehydration period, samples were taken to determine the water content of the embryos. Freezing was carried out in 2 ml cryovials by direct immersion in liquid nitrogen. After a minimum of one hour storage at -196°C, the embryos were rewarmed by plunging the cryotubes for 60 to 90 s in a 40°C water-bath and then transferred onto the culture medium for recovery. The cultures were kept under low light (2 $\mu E.m^{-2}.s^{-1}$, 12h/24 photoperiod) for two days and then placed under normal light conditions (44 $\mu E.m^{-2}.s^{-1}$, same photoperiod) for germination and further development. The culture temperature was 27 ± 1°C.

Freezing results are the average of 3 experiments with 3 replicates of at least 12 embryos each. Survival was observed 4 weeks after freezing. At that time, living embryos had produced their first pair of leaves.

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RESULTS

Untreated zygotic embryos extracted from yellow and green fruits showed 100% germination (Table 1 and 2). Water content varied with the maturity stage of the fruits from which the embryos were isolated (64% from green fruits, 42% from yellow fruits).

Embryos of coffee resisted freezing in liquid nitrogen only when dehydrated to water contents between 28.9% (Table 3) and 7.8% (Table 2). However, the rate of survival depended on the maturity stage of the fruits at the time embryos were isolated and on the water content of the embryos at the moment of freezing.

Embryos from immature (green) fruits of *C. arabica* were sensitive to air dehydration (Table 1), since after 30 min desiccation, survival decreased to 80% and fell to 52% after one hour desiccation. Embryos isolated from intermediate maturity (yellow) fruits of *C. arabica* showed 100% germination after 0 and 30 min desiccation (around 16% water content), but the germination rate decreased rapidly (to 55% and lower) when the dehydration period was increased (Table 2). Survival in liquid nitrogen was observed for desiccation durations ranging between 0.5 hour (16.4% water content) and 1.5 hours (7.8% water content). No survival was obtained for dehydration periods shorter than 0.5 hour (data not shown) and longer than 1.5 hours.

Even though embryos from mature (red) fruits of coffee dehydrated using the same process showed 100% survival after cryopreservation, contamination problems did not allow recovery of plantlets. At this maturity stage many fruits are burst or damaged by bacteria or by insects and even when using a stronger disinfection procedure, sanitary problems impeded the use of this material for conservation experiments.

In addition to *C. arabica*, freezing experiments were conducted with *C. canephora* and the hybrid arabusta. After 0.5 hour desiccation, embryos extracted from yellow fruits of *C. canephora* and arabusta had similar moisture contents around 28% (Table 3), whereas that of *C. arabica* was lower. Survival of desiccated control embryos was 100% with the three species. However, after cryopreservation, high survival rates (83 to 95%) were obtained with embryos of *C. arabica* and arabusta, whereas survival of *C. canephora* embryos was less than half of that of the two other species.

The relatively low survival rate of immature embryos of *C. arabica* (extracted from green fruits), desiccated for the optimal duration and frozen in liquid nitrogen, could be increased to levels comparable to that observed with more mature embryos (yellow fruits) by adding 10 to 100 $mg.l^{-1}$ GA_3 to the recovery medium (Table 4). However, their further development into whole plantlets was the most satisfactory after a treatment with 10 $mg.l^{-1}$ GA_3.

In all experiments, the further development of cryopreserved embryos was only slightly delayed, compared to that of unfrozen controls. In the case of embryos of *C. arabica* extracted from green fruits, this delay was reduced by the addition of 10 $mg.l^{-1}$ GA_3. Fully developed plantlets were obtained with all species.

DISCUSSION/CONCLUSION

During this study, an efficient cryopreservation method was set up for zygotic embryos of *Coffea*, since 41 to 96% survival of frozen embryos could be obtained, depending on the species.

This process is characterized by its simplicity, since the embryos were only submitted to a partial desiccation before their direct immersion in liquid nitrogen. It is similar to that used with zygotic embryos of several species (17). Indeed, zygotic embryos are usually desiccated in the air current of a laminar flow cabinet, down to optimal water contents of 10 to 16%, depending on the species. A comparable value was observed with embryos of *C. arabica* only. The embryos of *C. canephora* and arabusta are larger and may thus dehydrate more slowly. Therefore, their optimal dehydration period may be longer than that of *C. arabica* (0.5 hour).

Higher survival rates were obtained with more mature embryos, i.e. extracted from yellow fruits, compared with that extracted from green fruits. This may be due to the fact that immature embryos generally need to be grown on more sophisticated culture media (18) and not to a greater extent of damages caused to immature embryos by freezing in liquid nitrogen, in comparison to that observed with more mature ones. Indeed, the addition of GA_3, which is known to promote the growth of immature embryos (19) in the recovery medium allowed to increase the survival rate of immature embryos of *C. arabica* to a level comparable to that of more mature ones.

The extension of the dehydration period from 1.5 to 2 hours did not result in a further decrease in the water content of the embryos of *C. arabica* but induced a total loss of survival in cryopreserved embryos. This could be due to an increase in the damages caused to the tissues by this prolonged dehydration, as shown by the reduction in the survival rate observed with control embryos.

In conclusion, this preliminary study showed that it was possible to successfully cryopreserve zygotic embryos of *C. arabica*, *C. canephora* and the hybrid arabusta and to obtain the development of plantlets from frozen embryos. However, additional work is needed to improve these results: longer desiccation periods should be experimented with embryos of *C. canephora*. The further development *in vivo* of the plantlets should be monitored and the present technique refined and experimented with other *Coffea* species. The use of cryopreservation for the long-term preservation of coffee genetic resources may thus be foreseable in the near future.

ACKNOWLEDGEMENTS

The help of Mr. William Araya is gratefully acknowledged for his excellent technical assistance.

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Table 1: Moisture content and survival after dehydration (-LN) or dehydration and freezing in liquid nitrogen (+LN) of zygotic embryos of *C. arabica* isolated from green fruits.

Dehydration (hours)	Moisture content (%)	Survival (%)	
		-LN	+LN
0.0	64.2±1.2	100.0±0.0	0.0±0.0
0.5	21.2±1.9	80.0±0.0	50.0±4.0
1.0	10.1±0.9	52.6±7.4	34.2±5.4
1.5	7.8±1.3	25.0±5.3	13.9±2.7
2.0	5.0±2.1	5.5±2.7	0.0±0.0

Table 2: Moisture content and survival after dehydration (-LN) or dehydration and freezing in liquid nitrogen (+LN) of zygotic embryos of *C. arabica* isolated from yellow fruits.

Dehydration (hours)	Moisture content (%)	Survival (%)	
		-LN	+LN
0.0	41.9±0.5	100.0±0.0	0.0±0.0
0.5	16.4±0.4	100.0±0.0	96.0±4.0
1.0	8.4±0.3	55.5±2.8	41.6±6.2
1.5	7.8±1.1	27.7±2.7	19.4±2.8
2.0	7.7±1.5	8.3±0.0	0.0±0.0

Table 3: Survival after dehydration (-LN) or dehydration and freezing in liquid nitrogen (+LN) of zygotic embryos of *C. arabica*, *C. canephora* and the hybrid arabusta isolated from yellow fruits and dehydrated for 0.5 hour.

	Moisture content (%)	Survival (%)	
		-LN	+LN
C. arabica	15.8±0.26	100.0±0.0	95.8±4.1
C. canephora	28.9±2.9	100.0±0.0	41.6±2.2
arabusta	27.3±3.3	100.0±0.0	83.6±3.6

Table 4: Effect of GA_3 concentration in the recovery medium on the survival rate of embryos of *C. arabica* extracted from green fruits, dehydrated for 0.5 hour and frozen in liquid nitrogen.

GA_3 ($mg.l^{-1}$)	Survival (%)
0.0	52.1±4.2
10.0	75.0±0.0
33.3	75.0±8.3
100.0	83.3±8.3

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12. Somatic embryogenesis in grape

D.J. Gray

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1. Introduction

Grape (Vitaceae, *Vitis* spp.) is a deciduous temperate fruit crop of ancient origin. *Vitis* contains two subgenera, *Euvitis* Planch., the bunch grape species that all contain 38 somatic chromosomes, and *Muscadinia* Planch., the muscadine grapes with 40 somatic chromosomes (Einset and Pratt, 1975; Winkler *et al.*, 1974). Species in each subgenus are interfertile but are only partially fertile between subgenera (Jelenkovic and Olmo, 1969). Estimates of the number of *Euvitis* species range from 28 to 43. These are separated into an American group of 18 to 28 species, an Asian group of 10 to 15 species and a European or central Asian group of one species (*Vitis vinifera* L.) (Einset and Pratt, 1975). *Muscadinia* contains three species; *Vitis rotundifolia* Michaux., *Vitis munsoniana* Simpson, both native to the southeastern United States and *Vitis popenoeii* Fennell, native to central America, which comprise the muscadine grape varieties. With the exception of muscadine grapes, which are morphologically and genetically distinct, most cultivated grapes are either pure strains or hybrids of *V. vinifera* and account for the vast majority of world production. Hybrids of *Vitis labrusca* L., an eastern North American native (Galet, 1988), and *V. vinifera*, some with additional native species parentage, are in widespread production in the northeastern United States (Cahoon, 1986). Complex hybrids between *V. vinifera* and various native American species form a series of disease resistant French-American hybrids (Einset and Pratt, 1975). Certain species and interspecific hybrids are utilized exclusively as rootstocks. A number of other *Vitis* species are used in breeding programs to adapt high-quality *vinifera* germplasm to suboptimal environmental regions (Alleweldt and Possingham, 1988).

S. Jain, P. Gupta & R. Newton (eds.), *Somatic Embryogenesis in Woody Plants*, Vol. 2, 191–217.
© 1995 Kluwer Academic Publishers. Printed in the Netherlands.

Grape production (65 × 10^6 metric tons) exceeds that of any other temperate fruit crop and ranks after *Citrus* and banana among all fruit crops worldwide (FAO Production Yearbook, 1990). Grape surpasses all other fruit crops in value due to its multiple uses for fresh fruit, juice, jelly, raisins and wine. Wine production demonstrates the ultimate value of a fruit crop, with prices sometimes exceeding thousands of dollars per bottle.

1.1. *Conventional genetic improvement*

Although *V. vinifera* generally is considered to have the most desirable fruit quality, it is susceptible to many pests and diseases, including anthracnose, black rot, botrytis bunch rot, crown gall, downy mildew, eutypa dieback, various nematodes, phomopsis cane and leaf spot, phylloxera, Pierce's disease and powdery mildew. Hybridization with resistant species has been the only method available to produce resistant cultivars (Einset and Pratt, 1975; Galet and Morton, 1990). Other improvement objectives for grape include increasing fruit quality and yield.

However, improvement is slow and time-consuming because grape is heterozygous and exhibits pronounced inbreeding depression (Einset and Pratt, 1975). Therefore, an individual genotype cannot be reproduced by seed and all propagation of outstanding selections must be by vegetative means. The life cycle is relatively long, with a juvenility period ranging from one to six years, depending on environmental conditions and management practices (Einset and Pratt, 1975), such that fruit quality of progeny cannot be assessed for several years. Furthermore, resistance to many diseases cannot be confirmed until after several fruit-bearing years have passed. Another problem with the breeding approach, is that certain quality attributes of specific *vinifera* varieties, such as the color stability and flavor of "Cabernet Sauvignon", cannot easily be transferred to disease-resistant hybrids. Outstanding characteristics of varieties such as "Cabernet Sauvignon" were selected in ancient times, are genetically fixed, and are maintained by exclusive use of clonal propagation. Because of these obstacles to genetic improvement, alternative approaches to bypass sexual hybridization in order to add specific traits while maintaining the genetic integrity of such desirable varieties is especially attractive for grape.

1.2. *Genetic improvement through biotechnology*

For grape, *in vitro* genetic manipulation of somatic and/or zygotic cells holds great promise by either supplementing and enhancing existing breeding techniques or by circumventing conventional methods altogether. For example, somatic hybridization via protoplast fusion potentially might be utilized to circumvent the sterility inherent in crosses between *Euvitis* and *Muscadinia*, perhaps allowing seedlessness to be introduced into the former. While routine recovery of haploid and/or dihaploid plants via anther or ovule culture has not been accomplished in grape [haploid callus (Gresshoff and Doy, 1974) and plants (Zou and Li, 1981) were isolated reports], the potential of fertile dihaploids would be great, since all recessive genes would be expressed. Genetic mapping would be greatly simplified by use of such homozygous material. Genetic transformation offers significant opportunities for grape improvement by allowing specific traits to be introduced into otherwise traditional cultivars. Possible early applications of genetic transformation, include the addition of virus resistance by integration of viral coat protein genes (Nejidat *et al.*, 1990; Stark and Beachy, 1989), conferring resistance to insect pests by incorporation of genes that code for *Bacillus thuringensis* toxin (Barton *et al.*, 1987; Delannay *et al.*, 1989) and addition of genes to obtain herbicide tolerance (Oxtoby and Hughes, 1990; Shah *et al.*, 1986). Synthetic seed technology of grape, which would allow clonal germplasm of grape to be conserved in seed repositories, is relatively well-advanced as described below.

Implementation of these improvement strategies requires ability to manipulate and achieve regeneration from grape cells and tissues *in vitro*. In this chapter, the phenomenon of grape embryogenesis is reviewed in light of existing literature by exploring past and present approaches to culture initiation and maintenance, manipulation of embryo and plant development and specific uses for embryogenic culture systems.

2. Somatic embryogenesis

2.1. *Background and historical perspective*

Both embryogenic and, less commonly, organogenic regeneration pathways occur and previously were compared (Gray and Meredith, 1992). While only somatic embryogenesis is discussed here, it must be noted that difficulty in distinguishing between embryogenesis and organogenesis led to past confusion, since abnormal development during somatic embryogenesis can resemble shoot or root organogenesis. For example, examination of early literature concerning grape regeneration suggests that somatic embryogenesis was described as organogenesis in at least two instances. Shoot development from anther-derived callus of *Vitis thunbergii* Sieb. et Zucc. (Hirabayashi *et al.*, 1976) actually may have been an example of somatic embryogenesis. Although it is impossible to confirm, the regenerated structures resembled somatic embryos, based on an assessment of published photographs. Further, a paper by Favre (1977) is often mistakenly cited as an early report of shoot organogenesis, possibly due to the fact that the paper is in French, leading to misinterpretation by non-French speaking researchers. Translated to English, the critical text from this paper describes "bud formation that is first manifested by the appearance of small white organs. These develop into bipolar structures with an apical pole having more or less flattened pieces".

Table 1. Chronological listing of selected publications concerning grape somatic embryogenesis.

Date	Species/Variety	Explant[1]	Medium[2]	Auxin[3] (μM)	Cytokinin[4] (μM)	Culture age[5]	Germination treatment	Reference
1976	*V. vinifera* "Cabernet Sauvignon"	O-Nu	N	5–20 NOA	5–10 BA	NG	1 GA + 5 IP	Mullins and Srinivasan
1977	*Vitis* hybrid "Seyval Blanc"	S, L, F	MS	4.5 2,4-D	0.4 BA	6 mo.	none	Krul and Worley
1979	*V. vinifera* × *V. rupestris*	A	N	5 2,4-D	1 BA	NG	4C	Rajasekaran and Mullins
1980	*V. longii*, *V. rupestris*, *V. vinifera* "Grenache", *V. vinifera* × *V. rupestris*	A	N	5 2,4-D	1 BA	NG	4C	Mullins and Rajasekaran
1980	*V. vinifera* "Cabernet Sauvignon", "Grenache", *V. vinifera* × *V. rupestris*	O-Nu	N	5 NOA or 5 2,4-D then 10 NOA	1 BA	NG	Plants not obtained	Srinivasan and Mullins
1983	*V. longii*, *V. rupestris*, *V. vinifera* "Grenache", "Sumoll" × "Cabernet Sauvignon", *V. vinifera* × *V. rupestris*, *Vitis* hybrid "Villard Noir", "Villard Blanc", M.G. 60–44	A	N	5 2,4-D	1 BA	NG	NG	Rajasekaran and Mullins (a and b)
1985	*Vitis* hybrid "Seyval Blanc"	NG	NG	NG	NG	Indefinite	1 BA	Krul
1986	*V. vinifera* "Cabernet Sauvignon"	A	MS	4.5 2,4-D then 0.5 NAA	1 BA	NG	4C + cotyledon removal	Mauro *et al.*
1987	*V. longii*	A and Ov	MS	5 2,4-D	1 BA	20 mo.	1 BA or dehydration	Gray (b and d); Gray and Mortensen
1988	*V. rupestris*, *V. vinifera*	L	N	4.5 NOA	0–4.4 BA	NG	none or various growth regulators	Stamp and Meredith (a)
1988	*V. rupestris*, *V. vinifera* "Cabernet Sauvignon", "Cardinal", "Grenache", "Sauvignon Blanc", "Thompson Seedless", "White Riesling", *V. vinifera* × *V. rupestris*	A	N	4.5 2,4-D or 4.5–7 NOA	0.9–4.4 BA	NG	none or various growth regulators	Stamp and Meredith (a)
1988	*V. longii*, *V. vinifera* "Chardonnay", "French Colombard", "Grenache", "White Riesling"	Z	N	4.5 2,4-D or 4.4–18 NOA	0–4.4 BA	12 mo.	none or various growth regulators	Stamp and Meredith (b)
1989	*V. longii*, *V. vinifera* "Thompson Seedless"	A, Ov, L	MS	5 2,4-D	1 BA	48 mo.	1 BA or dehydration	Gray
1989	*Vitis* hybrids	O	MS	10 IAA[6]	1 BA	42 mo.	1 BA or dehydration	Gray
1989	*V. vinifera* "Koshusanjaku"	L	N, MS	5–10 2,4-D w./wo. transfer to 1 2,4-D	5–10 BA or 5–10 TDZ or 5–10 KT	24 mo.	none	Matsuta and Hirabayashi
1992	*V. rotundifolia* five varieties	O then Z	NN	1 mg/l NOA	0.2 mg/l BA	12 mo.	MS + 1 μM BA	Gray
1992	*V. aestivalis*, *V. vinifera* "Koshusanjaku"	L	NN	10 μM 2,4-D or 2,4,5-T	10 μM KT	NG	NG	Matsuta
1993	*V. rotundifolia* 11 varieties	O then Z	NN	1 mg/l NOA	0.2 mg/l BA	NG	MS + 1 μM BA	Gray and Hanger
1993	*V. rupestris*	L	MS	0.1 or 1 mg/l 2,4-D	1 mg/l BA	24 mo.	4C + 1 mg/l BA + 1 mg/l IBA	Martinelli *et al.*
1993	*V. rotundifolia* 2 varieties	L	NN	9 μM 2,4-D, then 10.7 μM NAA	4.4 μM BA, then 0.9 μM BA	NG	1 μM BA	Robacker
1993	*V. vinifera* "Riesling"	A, F	NN	4.5 μM 2,4-D	10 μM BA or 10 μM TDZ	NG	NG	Harst-Langenbucher and Alleweldt

[1] A – anthers; F – floral tissue; L – leaves (includes use of lamina and/or petiole); NG – explant not given; O – fertilized ovule; O-Nu – unfertilized ovule cultured – nucellus confirmed to be source of embryogenic cells; Ov – unfertilized ovaries; S – shoot tip; Z – zygotic embryos.
[2] MS – Murashige and Skoog (1962); N – Nitsch and Nitsch (1969). Note that original medium formulae were often modified.
[3] 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indole-3-acetic acid; NAA – α-naphthaleneacetic acid; NOA – naphthoxyacetic acid.
[4] BA – 6-benzyladenine; KT – N-(2-chloro-4-pyridyl)-N'-phenylurea; TDZ – thidiazuron – N-(1,2,3-thiadiazol-5-yl)-N'-phenylurea.
[5] Culture age – Time period over which embryogenic cultures could be maintained.
[6] Embryogenic culture initiation medium with IAA also contained 1 μM gibberellic acid.

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The author states that these structures "resembled the plantlets found in seeds". It is clear from this description that somatic embryos were obtained (compare with description of somatic embryogenesis in the section below).

Grape somatic embryogenesis was first unequivocally reported by Mullins and Srinivasan (1976), who cultured unfertilized ovules of *V. vinifera* "Cabernet Sauvignon" in liquid medium containing napthoxyacetic acid (NOA) and 6-benzyladenine (BA). Proliferating nucellus-derived callus was extruded through the ovular micropyle and eventually gave rise to somatic embryos. Somatic embryos germinated into plants when transferred to agar-solidified medium that contained gibberellic acid (GA_3) and 2-isopentenyladenine (2-iP). Long term maintenance of cultures was not described. Krul and Worley (1977) documented somatic embryogenesis from calli derived from leaf, petiole and stem segments of the French-American hybrid "Seyval". This culture protocol differed from that of Mullins and Srinivasan (1976), since solidified medium was used that contained 2,4-dichlorophenoxyacetic acid (2,4-D) to induce callusing. Embryos developed after transfer of callus to medium containing α-naphthaleneacetic acid (NAA) and BA. Embryos germinated into plants after transfer to medium devoid of growth regulators. The original embryogenic line could be maintained indefinitely through secondary embryogenesis on growth regulator-free medium. A US patent describing this method of embryogenesis and plant production was eventually awarded (Krul, 1985). In the intervening years, research on grape somatic embryogenesis was reported by a number of researchers (Table 1).

2.2. *Culture initiation*

Genotypic specificities. Initiation of embryogenic cultures in grape is highly genotype-dependent, with all but a few successful reports emanating from only four species: *Vitis aestivalis* Michx., *V. longii* Prince, *V. rotundifolia*, *V. rupestris* Scheele or *V. vinifera* (Table 1). Most hybrids that produced embryogenic cultures are composed of at least one of these species. For example, *Vitis* hybrids "Seyval Blanc", "Villard Blanc" and "Villard Noir" have both *V. rupestris* and *V. vinifera* parentages. Additionally, genotypic specificities are noted within species. This is illustrated by *V. vinifera*, where very few cultivars were documented to produce somatic embryos. Genotypic specificity is currently an obstacle to implementing a broad range of biotechnological procedures, since regenerative cultures are not yet available for many important cultivars.

Sex of donor vine. Sex of the donor vine was suggested to be a contributing factor in the ability of particular explants to yield embryogenic cultures. Rajasekaran and Mullins (1983b) noted that the propensity of anthers from male vines to form somatic embryos was greater than that of hermaphroditic vines and that anthers from female vines did not form somatic embryos at all. Treatment of a male vine with cytokinin to feminize the flowers inhibited

anthers while it stimulated the ovules' ability to produce somatic embryos (Rajasekaran and Mullins, 1983b; Srinivasan and Mullins, 1980). However, Gray and Mortensen (1987) found that both anthers and ovaries of untreated female *V. longii* "Microsperma" yielded embryogenic cultures. Therefore, the importance of sex expression on the ability of an explant to produce somatic embryos is somewhat unclear. However, growth responses of tissues from male and female vines clearly can be manipulated with growth regulators.

Explant source. Explant tissue type and condition are critical to successful culture initiation. Most successful attempts have relied on floral tissues, particularly anthers or unfertilized ovules (with the nucellus documented to be the source of embryogenic cells in the latter); however, leaves, shoot tips and zygotic embryos were also utilized (Table 1). Recently, preculture of fertilized ovules was employed to obtain immature zygotic embryos, which were used as explants to develop embryogenic cultures of *V. rotundifolia* (Gray, 1992). Subsequently, it was demonstrated that ovules cultured at maturities ranging from 20 to 40 days after pollination were equally effective in development of embryogenic cultures from 11 *V. rotundifolia* varieties (Gray and Hanger, 1993). Explant developmental stage as well as preconditioning may be important in certain instances. For example, anthers approximately 0.5 mm long were optimal for inducing embryogenic cultures of *V. vinifera* "Cabernet Sauvignon" (Mauro *et al.*, 1986). Anther translucence and color (translucent yellow-green) served as an indicator for determining the proper stage of both anther and unfertilized ovary explants (Goussard *et al.*, 1991; Newton and Goussard, 1990). Only leaves 1.5 to 5.0 mm in length produced embryogenic cultures for several varieties (Stamp and Meredith, 1988a). For anthers, chilling at 4°C for 72 h (Rajasekaran and Mullins, 1979) or 96 h (Harst-Langenbucher and Alleweldt, 1993) prior to culture resulted in an increase of callusing and embryogenesis when compared to no cold treatment. Perhaps other, as yet untested, methods of pretreating explants would allow extension of the embryogenic response to additional species and cultivars.

Origin of embryogenic cells. The specific origin of embryogenic cells in primary explants of grape is obscure. Rajasekaran and Mullins (1983a) suggested that anther somatic tissues and not gametic cells gave rise to embryogenic callus, since haploids and homozygous diploids were not obtained among regenerants. This observation was reinforced by Newton and Goussard (1990) who described embryogenic callus growing from the filament, interlocular parenchymatous tissue and/or anther wall epidermis in *V. rupestris*. Embryogenic callus appeared to arise from the nucellus in unfertilized ovules (Mullins and Srinivasan, 1976); whereas most polyembryos from fertilized ovules were of zygotic embryo origin (Durham *et al.*, 1989). In cultured leaves, embryogenic callus arose from mid-vein and adjacent lamina tissue

(Stamp and Meredith, 1988a). However, these studies did not pinpoint the actual tissue origin(s) of the embryogenic cells.

Culture medium. Basic embryogenic culture medium formulations have received relatively little attention. We are aware of only two, MS medium (Murashige and Skoog, 1962) and Nitsch's medium (Nitsch and Nitsch, 1969), or modifications thereof, that were successfully utilized (Table 1). However, details of medium formulation differ among various researchers. For example, Mullins and coworkers (Mullins and Srinivasan, 1976; Rajasekaran and Mullins, 1979; Mullins and Rajasekaran, 1980) utilized a liquid medium system, whereas others used agar-solidified medium. Stamp and Meredith (1988a) compared both liquid and solid methods for leaf culture and found only the latter to be suitable. Other media addenda are similar to those commonly used in cell culture of many other species, such as sucrose at 20 to 60 $g \cdot l^{-1}$. Study of the effects of 1 $mg \cdot l^{-1}$ adenine, 100 $mg \cdot l^{-1}$ glutamine and 10 $mg \cdot l^{-1}$ phenylalanine on embryogenesis from anthers showed that a combination of all three was better than any two or none at all (Mauro *et al.*, 1986).

Generally, the phenoxy-auxins 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) or NOA, combined with the cytokinin BA, are utilized to initiate embryogenic cultures (Table 1); however, indole-3-acetic acid (IAA) combined with GA_3 was used to produce long-lived embryogenic cultures from fertilized ovules (Gray, 1989). Presumably, in the latter instance, embryogenic cultures stemmed from immature zygotic embryos (i.e., from preexisting embryogenic cells) and an induction stage was unnecessary. Cytokinins thidiazuron (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (KT-30) were shown to be superior to BA for leaf explants of *V. vinifera* "Koshusanjaku" (Matsuta and Hirabayashi, 1989) whereas more embryogenic cultures were obtained with BA than TDZ for anther explants of *V. vinifera* "Riesling", suggesting either an explant and/or genotypic difference in response to cytokinin.

Culture conditions. Reports in the literature of physical culture conditions vary considerably to the degree that specific requirements are uncertain. For example, Stamp and Meredith (1988a) cultured anthers in total darkness, whereas a 16/8 h light dark photoperiod was used for leaf explants. For anthers of *V. vinifera* "Riesling", an incubation temperature of 27°C resulted in increased embryogenesis when compared to 15°C or 32°C temperatures (Harst-Langenbucher and Alleweldt, 1993).

Examples of culture protocols. Two distinct culture protocols are given that have proven to be useful for multiple cultivars. *Protocol* 1. This culture protocol, described by Goussard *et al.* (1991), utilized unfertilized ovaries, obtained from inflorescences forced from dormant wood cuttings and was similar to that previously described by Mullins and Srinivasan (1976). Dormant canes 40 cm in length were treated with 2% Captan and conveniently stored for several months until needed at 2–3°C. The bases of canes sectioned into 12–15 cm lengths, each with 2 internodes and three buds, were placed into 80 ml water and kept at 28–30°C under constant illumination. After bud burst at 5–8 days, all vegetative organs were removed from shoots bearing inflorescences to encourage flower development, which were harvested when anthers were translucent yellow-green in color (maturation status of microspores was not considered). Twenty ovaries were cultured per 100 ml Nitsch and Nitsch liquid medium with 5 μM BA, 2.5 μM NOA and 2.5 μM 2,4-D. Incubation was at 80 rpm, 25°C in dark. After 30 days, tissues were transferred to the same medium, except that it was solidified, and incubated as before for 30 days. Embryogenesis occurred after transfer to the same medium, but lacking growth regulators. This procedure was used to routinely obtain somatic embryogenesis from seven *V. vinifera* varieties (P.G. Goussard, personal communication). *Protocol* 2. A multi-step solid medium culture system was used to induce embryogenesis from laminae and petioles of two *V. rotundifolia* varieties (Robacker, 1993), a species that previously was regarded as recalcitrant. Following culture on Nitsch's medium containing 9 μM 2,4-D and 4.4 μM BA, explants were transferred to medium with 10.7 μM NAA and 0.9 μM BA to encourage callusing. The cultures were then transferred to growth regulator-free medium. All cultures were maintained under a 16/8 h light/dark cycle at 27–30°C. Up to 19% of laminae and 91% of petioles eventually produced embryogenic cultures. Of the embryogenic cultures obtained, 10% of those from laminae and 50% of those from petioles were noted on the second medium; however, most embryogenic cultures did not emerge from the callus mass until after transfer to basal medium. Thus, this study suggested that an intermediate treatment to encourage cell division ultimately resulted in a higher frequency of embryogenesis.

Culture morphology. Embryogenic callus or embryos typically emerged some time after nonembryogenic callus had formed on initiation medium. Fig. 1A shows the emergence of embryogenic callus from a callused, necrotic leaf explant of *V. vinifera* "Thompson Seedless". Embryogenic callus is granular and white to yellow in color. Often a highly heterogenous callus, composed of white, yellow and/or green, soft, friable, dry to wet sectors develops from the explant (Gray and Mortensen, 1987). Cells of the friable callus were highly variable in shape and size. Often, this initial callus declines and becomes brown prior to emergence of embryogenic callus and somatic embryos (Gray, 1992). In rarer instances, embryogenic callus emerged directly from seemingly uncallused explants (D.J. Gray, personal observation). Embryogenic callus was typically white to pale yellowish and emerged from distinct sectors of the heterogenous callus. Newly-initiated embryogenic callus of grape was composed of clusters of small isodiametric cells, interspersed with larger vacuolate cells (Gray and Mortensen, 1987). The embryogenic cells were easily identified by their prominent nuclei and nucleoli as well as

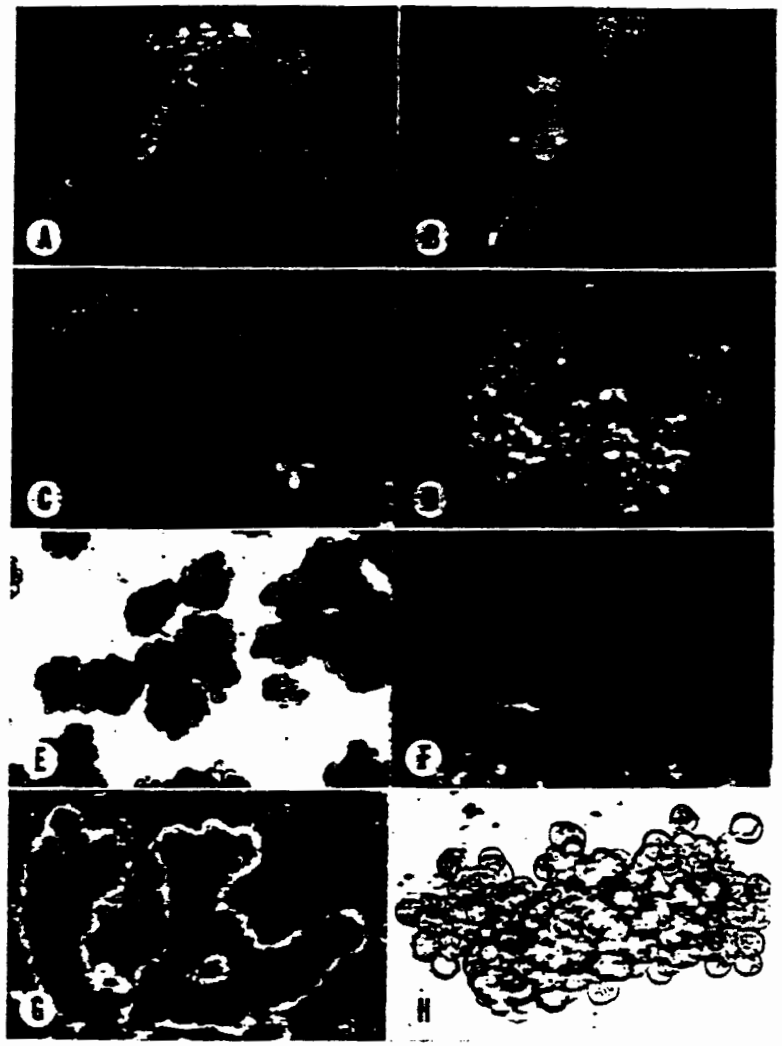


Figure 1. Somatic embryogenesis in grape. (A) White granular embryogenic callus emerging from cultured leaf of *Vitis vinifera* "Thompson Seedless". Note necrotic edge of lamina visible at bottom left (× 10). (B) Direct somatic embryogenesis from hypocotyl-radicle boundary of previously-formed embryo (× 14). From Gray and Mortensen (1987). (C) Somatic embryogenesis occurring from sectors of heterogenous callus maintained on medium with 2,4-D and BA (× 20). (D) Non-sectored embryogenesis from homogenous callus maintained on growth regulator-free medium (× 8). (E) Embryogenic cell clusters that compose homogenous embryogenic callus (× 85). (F) Embryo development on homogenous embryogenic callus (× 15). G) Organized embryogenic cell clusters typical of cultures maintained on medium with 2,4-D and BA (× 50). H. Uniform embryogenic cell culture typical of cultures maintained on growth regulator-free medium (× 160).

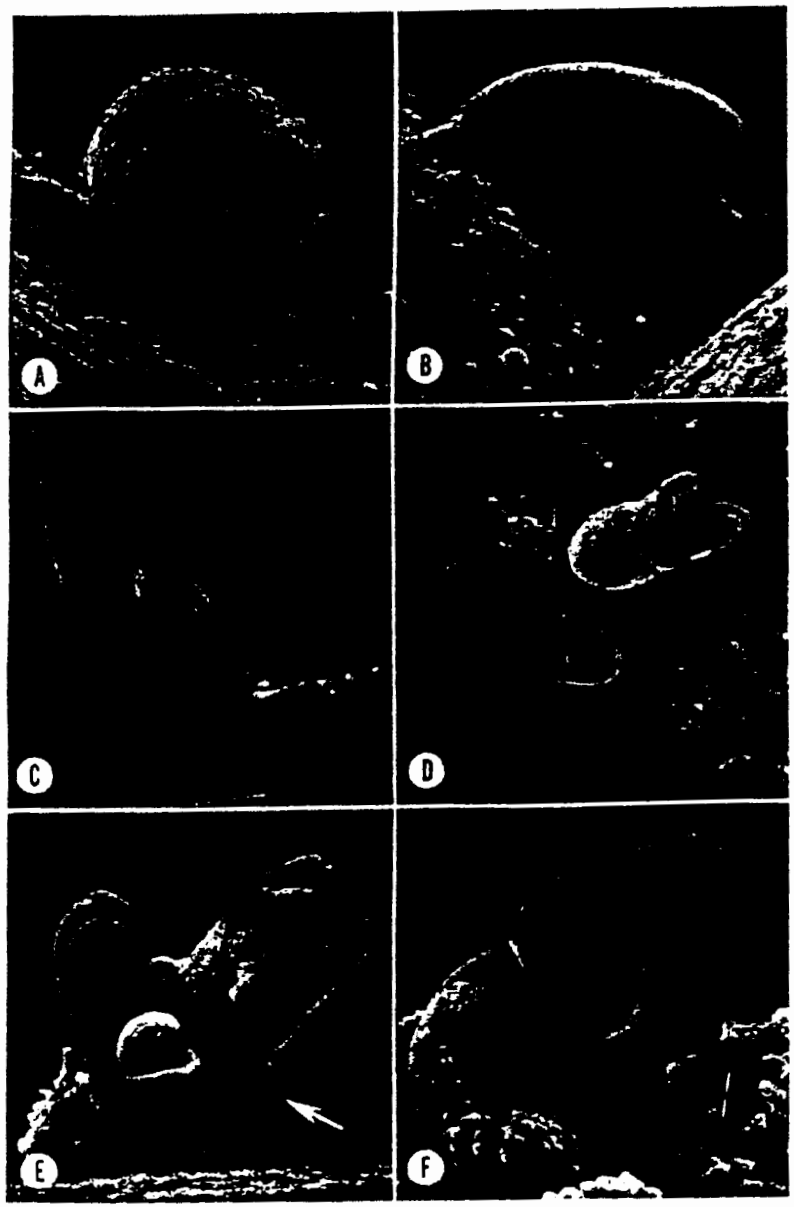
densely stained cell walls and cytoplasm (Krul and Worley, 1977). Embryogenic culture material typically contained distinct cell clusters, somatic embryos and highly abnormal somatic embryos that appeared as amorphous masses of white, compact, highly organized tissue embedded in a watery matrix (Gray and Mortensen, 1987; Gray, 1992). Microscopic examination revealed that the individual cells of embryogenic callus were highly uniform, being small, densely cytoplasmic with amyloplastids and lipoplastids (Gray, 1992).

2.3. *Culture maintenance*

Early reports by Mullins and coworkers (Table 1) suggested that somatic embryogenesis was a terminal event in their culture system and that regenerative cultures could not be maintained. However, Krul and Worley (1977) demonstrated that cultures could be maintained for at least six months and, subsequently, "indefinitely" (Krul, 1985). A *V. longii* cell line, homogenously composed of embryogenic-type cells, has been in continuous culture for eight years, although few somatic embryos now develop (D.J. Gray, unpublished data). Other documented time periods for culture maintenance have ranged from 12 to 48 months (Table 1). Thus, under certain conditions, grape embryogenic cultures are quite long-lived. Grape embryogenic cultures were maintained via two distinct methods: direct secondary embryogenesis or indirect embryogenesis via culture of embryogenic cells.

Direct secondary embryogenesis. Direct secondary embryogenesis was reported for several grape species and cultivars (Gray and Mortensen, 1987; Krul and Worley, 1977; Rajasekaran and Mullins, 1979; Srinivasan and Mullins, 1980). However, its use for culture maintenance was adequately explored only for *Vitis* hybrid "Seyval Blanc" (Krul, 1985; Krul and Worley, 1977) and *V. longii* "Microsperma" (Gray and Mortensen, 1987). With this regeneration pathway, new somatic embryos grew directly from previously-formed somatic embryos. Interestingly, the preponderance of new embryos emerge from at or near the hypocotyl-radicle boundary as illustrated in Fig. 1B (Gray and Mortensen, 1987; Krul, 1985). Secondary embryos first appear as clavate structures on the primary embryo surface (Fig. 2A) and undergo morphological development typical of zygotic embryos (Fig. 2B), eventually becoming fully mature in appearance (Fig. 2C). All stages of embryogeny are often present at a given time (Fig. 2D). A distinct suspensor is not obvious; however, secondary embryos are barely connected to primary embryos, suggesting a suspensor-like attachment. It is not known whether or not cells predisposed to develop into embryos exist at the hypocotyl-radicle boundary or if, perhaps, endogenous hormone levels that favor induction of embryogenic cells are optimized at this location.

For direct secondary embryogenesis, cultures were maintained by transferring either single embryos or clumps of embryos. After 4–8 weeks, secondary



embryos were harvested and transferred to fresh medium. While both MS and Nitsch's media were used for maintenance, growth regulators were not required for continuous secondary embryogenesis to occur (Gray and Mortensen, 1987; Krul, 1985). Ability to undergo direct secondary embryogenesis in the absence of exogenously-supplied growth regulators appears to be possible in many, if not all, grape genotypes from which somatic embryogenesis has been induced (D.J. Gray, personal observation). However, propagation via direct secondary embryogenesis is relatively unwieldy and increase of embryogenic culture mass is slow, when compared to indirect embryogenesis from embryogenic cell culture. Furthermore, certain biotechnical applications, such as protoplast fusion and genetic transformation may be accomplished more easily with cell cultures.

Indirect embryogenesis. For indirect embryogenesis, cell cultures were maintained by either of two methods: 1) continual transfer on medium containing auxin with or without cytokinins, or 2) subculture on medium devoid of growth regulators.

Gray and Mortensen (1987) noted that callus maintained on medium with 2,4-D and BA continually sectored into embryogenic and non-embryogenic portions (Fig. 1C), which required careful separation during transfer; whereas Stamp and Meredith (1988a) and Matsuta and Hirabayashi (1989) did not report this phenomenon. The latter researchers reported that embryogenic callus was maintained by omitting vitamins, glycine and inositol from maintenance medium.

Embryogenic cell culture on growth regulator-free medium was first reported by Gray (1989) for *V. longii*, *V. vinifera* "Thompson Seedless" and three hybrid grape lines. The medium utilized was solidified MS with 6% sucrose and 0.2% activated charcoal. The latter ingredient was added to reduce culture browning. The same medium, except modified to contain $\frac{1}{4}$ of the normal MS nitrogen concentration, subsequently was utilized to maintain embryogenic cell cultures of several members of subgenera *Euvitis* and *Muscadinia*. Culture growth and maintenance was enhanced by utilizing high medium-to-headspace volume ratios in culture vessels, low light intensities and careful discrimination and selection of embryogenic cell type during transfers (Gray and Benton, 1991). Subsequently, embryogenic cultures of

Figure 2. Direct (A-D) and indirect (E-F) somatic embryogenesis in *Vitis longii*. (A) Globular embryo on somatic embryo hypocotyl (× 290). (B) Late torpedo-stage embryo on somatic embryo hypocotyl (× 140). (C) Mature embryo on germinated somatic embryo (× 20). (D) Globular, heart and late torpedo stage embryos growing from somatic embryo (× 40). (E) Four embryos at different stages and cotyledonary abnormalities growing from callus. Note clockwise from globular embryo (arrow): heart stage embryo with two cotyledons, torpedo stage with three cotyledons and mature embryo with four cotyledons (× 36). From Gray and Mortensen (1987). (F) Mature stage embryo growing from callus (× 50).

five *V. roundifolia* varieties were developed utilizing this culture system (Gray, 1992). Selection of embryogenic cultures is accomplished by manually isolating the embryogenic cell masses and/or somatic embryos from initial embryogenic calluses. It was also possible to recover embryogenic cell culture lines from the watery residual material formed during direct somatic embryogenesis. This watery material was "scraped" from the agar surface and combined into small clumps, which would develop increasingly more cellular material and somatic embryos through subsequent transfers as described below. The embryogenic cultures of grape produced with this system were termed "perennial" due to their remarkable longevity of several years (Gray and Benton, 1991), in contrast to many other embryogenic culture systems (i.e., Gray *et al.*, 1993, for *Cucumis melo* L.) in which embryogenic potential declines rapidly over time.

Development of perennial-type embryogenic cell cultures that persist on growth regulator-free medium was dependent on stringent selection criteria utilized during transfer. In newly-initiated cultures, embryogenic cell masses were carefully separated from the adjacent tissue. All other material, including somatic embryos beyond the globular stage were excluded. Embryogenic cell material was combined into single clumps of approximately 3 mm in diameter. Selection of similar material and exclusion of somatic embryos was continued on successive transfers until a distinct cell line was obtained that was homogenously composed of proliferating cell clusters and young somatic embryos (Fig. 1D, E). Torpedo and cotyledonary-stage embryos were obtained by allowing globular embryos to remain in the culture mass through successive transfers (Fig. 1F); this resulted in masses of singulated embryos. As with direct secondary embryogenesis, somatic embryos that developed in this culture system passed through typical embryogenic stages (Figs. 2E, F). Utilizing these selection criteria, we developed perennial lines of over twenty species, hybrids and cultivars (D.J. Gray, unpublished data).

Comparison of cultures maintained on the aforementioned medium with or without 5 μM 2,4-D and 1 μM BA showed clear differences in morphology of embryogenic cell clusters (compare Figs. 1G and H). Embryogenic cell clusters from the heterogenous cultures that developed on medium with growth regulators were highly organized and embryo proliferation appeared to originate by a process of budding (Fig. 1G), whereas, cultures maintained on growth regulator-free medium were composed of small, relatively unorganized clusters of embryogenic cells (Fig. 1H) in which somatic embryos appeared to arise by organized divisions of single cells (Fig. 3A). Furthermore, only those cultures maintained without growth regulators persisted and could be regarded to be the "perennial" type.

It is unclear whether prolonged culture of embryogenic cells will result in mutation or other genetic abnormalities in grape as noted for some crops. The opportunity to maintain cultures with or without growth regulators may allow us to better define the effects of these substances on genetic stability. Embryo development from embryogenic cell cultures tended to decline

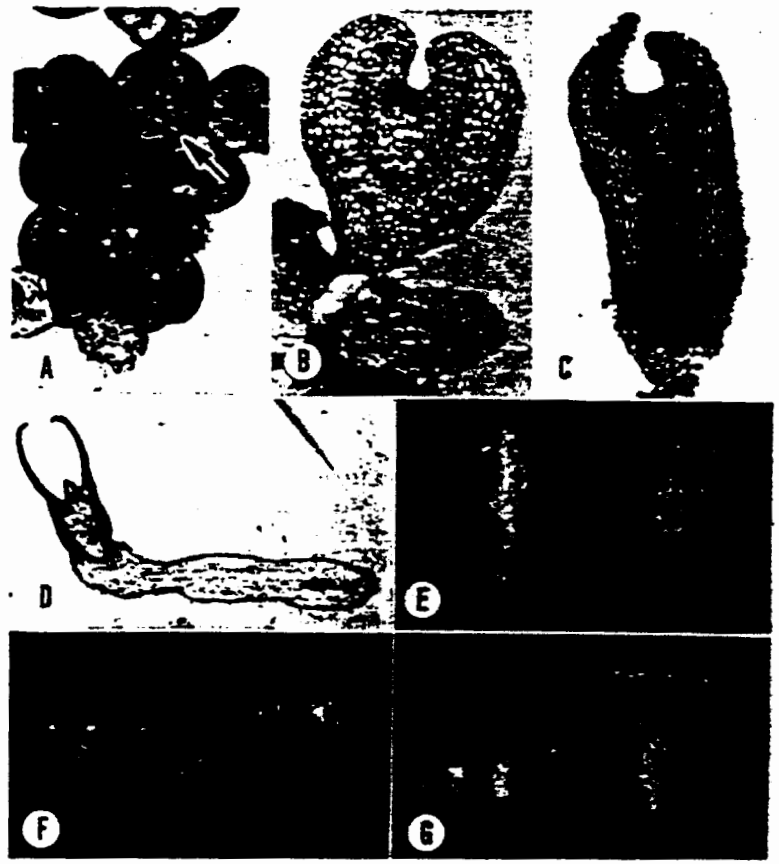


Figure 3. Somatic embryo development in *Vitis longii* "Microsperma", *Vitis roundifolia* "Carlos" and *Vitis longii* "Thompson Seedless". (A) Three cell-stage somatic embryo (arrow) in embryogenic cell cluster of "Thompson Seedless" (× 1200). (B) Heart-stage somatic embryo of "Microsperma" (× 210). From Gray and Mortensen (1987). (C) Torpedo-stage somatic embryo of "Thompson Seedless" (× 70). (D) Germinated somatic embryo of "Thompson Seedless" (× 14). (E) Comparison of zygotic embryo (left) with somatic embryo (right). Note flattened appearance of zygotic embryo (× 15). (F) Morphologically correct somatic embryo development from cell cultures of "Thompson Seedless". Note suspensors at base of embryos (× 80). (G) Abnormal cotyledon development in somatic embryos from cell cultures of "Carlos". Note early development of fused cotyledons in second embryo from right. From Gray (1992).

gradually over prolonged time periods in perennial cultures; however, the typical embryogenic cell morphology did not change, suggesting possible emergence of mutant cell lines. We have never observed the development of non-embryogenic-type callus from this system. Embryo development often could be reinitiated from cell cultures that are at least nine years old by providing a pulse on any of the initiation media described above (D.J. Gray, unpublished data); this suggests that stable embryogenic cells may persist over very long time periods. Further support for genetic stability of somatic embryos comes from studies of somatic embryo-derived poinsettia (*Euphorbia pulcherrima* Willd ex Klotzsch) plants obtained from genetically unstable long-term suspension cultures, which were morphologically uniform and cytogenetically normal (Geier *et al.*, 1992). The issue of genetic stability is further explored below under "Growth in the Field".

2.4. *Embryo development*

Somatic embryos appeared to develop from single embryogenic cells via a suspensor (Figs. 3A, B, F) (Gray and Mortensen, 1987; Krul and Worley, 1977). Somatic embryos passed through recognizable developmental stages, possessed normal epidermal, cortical and vascular tissues and tended to be opaque white in color from the early torpedo stage to germination (Figs. 2A-F and 3A-G). Somatic embryos anatomically and morphologically faithful to all zygotic embryo stages were commonplace; however, a number of developmental abnormalities often occurred. The most common abnormality was the development of more than two, often fused, cotyledons (Figs. 2E and 3G) or somatic embryos that were larger or less flattened than zygotic embryos (Fig. 3E) (Gray and Mortensen, 1987). The development of fused cotyledons could be visualized in developing embryos at a very early stage (note second embryo from left in Fig. 3G). Differences in size and shape of somatic vs. zygotic embryos is likely due to absence of developing seed tissues, which exert a profound influence on zygotic embryo morphology. More subtle developmental abnormalities, such as no-to-poor development of the shoot apex (Faure, 1990) also occurred. These abnormalities were not likely to be related to genetic changes, since plants of normal appearance were recovered from such embryos.

Histochemical staining showed that starch [determined with the PARS reaction (amyloplasts stain pink) in tissue that was fixed, dehydrated and embedded in water-soluble plastic resin and iodine (amyloplasts stain black) in fresh specimens] and storage proteins [determined with napthol blue-black staining (protein deposits stain black) in embedded material] were present in somatic embryos, but did not exist in appreciable amounts (Fig. 4A) (Gray, 1988; Gray and Mortensen, 1987). However, lipids, which were lost during conventional processing of specimens for histochemical analysis into water-soluble plastic resin, were abundant in fresh material when stained with osmium tetroxide or sudan black-B (lipoplasts stain black) (Fig. 4B)

Figure 4. Histochemical localization of storage compounds in somatic embryos of *Vitis vinifera* "Thompson Seedless". (A) Storage proteins identified via staining with napthol blue-black (large arrows) and starch identified via the PARS reaction (small arrow) are not abundant in mature somatic embryos (× 1400). (B) Profuse lipids emerge from crushed cotyledon stained with sudan black-B (× 140).

(Gray, 1988). Grape is an oil seeded crop and it appears that the somatic embryos are faithful to zygotic embryos in this regard, although analytical comparisons of lipid type and amount have yet to be accomplished.

2.5. *Embryo germination and plant development*

At least some degree of plant recovery was possible from the first report of grape somatic embryogenesis (Mullins and Srinivasan, 1976). However, actual plant recovery rates were low and specific germination requirements varied widely among subsequent reports. Faure (1990) noted that, although 27% of *V. rupestris* somatic embryos had a morphology and anatomical structure identical to that of zygotic embryos, only 3% germination and development into plants was obtained. Examination of the literature shows that somatic embryos were often subjected to a pretreatment to obtain germination (Table 1). Pretreatments included cold stratification and addition of auxins, cytokinins, or gibberellic acid. In rarer instances, no treatment at all was required to induce germination and recover plants. However, somatic embryos produced on growth regulator-free medium always required some type of pretreatment to germinate (Gray, 1987c, 1989; Gray and Mortensen, 1987; Krul, 1985). To better understand germination requirements of grape somatic embryos, it is useful to consider germination of seeds. Grape seeds exhibit a type of dormancy that is alleviated by cold stratification and they germinate after a prolonged treatment at 4°C (Flemion, 1937). Grape somatic embryos also are dormant since germination is improved by cold treatments (Gray and Mortensen, 1987). Where no pretreatment was required for grape somatic embryo germination, culture systems that utilized auxins with or without cytokinins were used to produce somatic embryos (Table 1). Thus,

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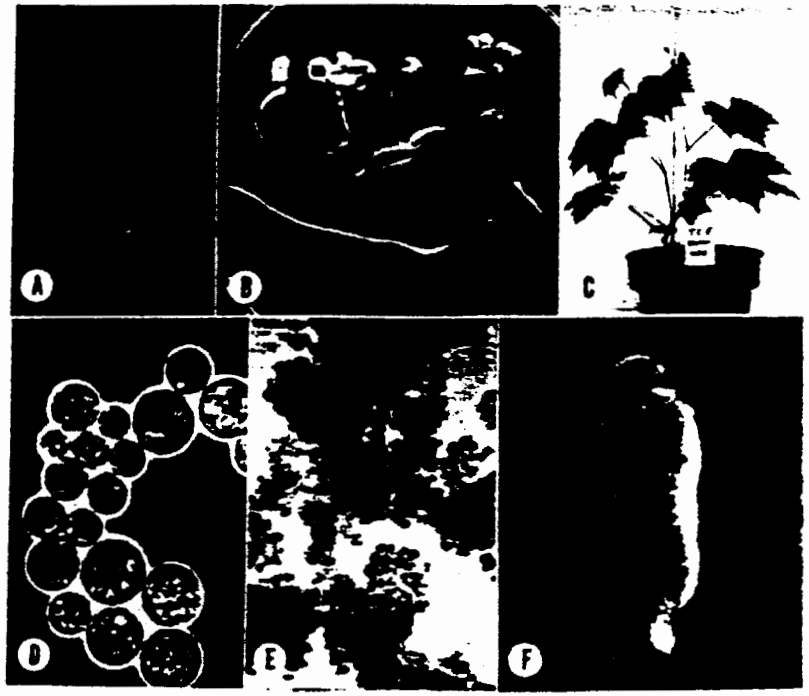

Figure 5. Plant recovery from somatic embryos and applications of somatic embryogenesis. (A) Dehydrated somatic embryo of *Vitis vinifera* "Thompson Seedless". Note shrivelled, discolored appearance (× 14). (B) Effect of growth regulators and dehydration on germination of "Thompson Seedless" somatic embryos. From left: no growth regulator (control), GA_3, ABA, BA. Bottom: dehydration (× 1). (C) Plant derived from somatic embryo of *Vitis rotundifolia* "Welder" (× 0.2). (D) Protoplasts derived from embryogenic cells of "Thompson Seedless" (× 1300). (E) Transient expression of GUS in embryogenic cell cultures of "Thompson Seedless" via particle bombardment. Dark areas denote sites of intense blue GUS reaction (× 40). (F) Transient expression of GUS in a "Thompson Seedless" somatic embryo. The entire side of the embryo facing particle discharge became intense blue in coloration (× 25).

these embryos were, in essence, subjected to a dormancy-breaking pretreatment.

The grape embryogenic culture system is the most studied example of somatic embryo dormancy. Although grape somatic embryos become very well developed and are morphologically similar to zygotic embryos, they germinate poorly. This demonstrates that the block to germination is at the physiological level and is not due to problems intrinsic to somatic embryo ontogeny. Studies of ABA concentration in grape embryogenic cultures showed a rapid increase during embryo development, which reached a peak

Table 2. Comparison of dehydration and benzyladenine (BA) for inducing germination in grape somatic embryos.[1]

Treatment[2]	Percent germination response[3]			
	Hypocotyl	Root	Cotyledon	Shoot
Dehydration	77	68	65	34
0.5 uM BA	100	92	98	12
Control	76	88	36	0

[1] From Gray (1989).
[2] Well-developed embryos were either dehydrated for 21 days at 70% RH and 27°C, placed directly on medium with BA, or placed on basal medium (control).
[3] Germination response was based upon either enlargement and greening of hypocotyls and cotyledons or emergence of roots or shoots.

at maturation (Rajasekaran *et al.*, 1982). Cold stratification of somatic embryos resulted in a rapid decrease in ABA concentration. Exogenously supplied ABA inhibited somatic embryo germination (Gray, 1989). ABA is implicated as a controlling factor of dormancy in many types of seeds (Bewley and Black, 1985). Contrary to ABA, exogenously supplied GA_3 induced grape somatic embryo germination and the concentration of endogenous GA_3-like compounds increased during cold stratification (Pearce *et al.*, 1987; Takeno *et al.*, 1983). These studies illustrate endogenous control of embryogenesis and germination, whereby ABA inhibits precocious germination and thus promotes normal development while GA_3 promotes germination. Exogenously-supplied ABA was shown to increase plant recovery in one line (Goebel-Tourand *et al.*, 1993) but not in others (Gray, 1989). Another related study showed that embryogenic suspension cultures of a *V. vinifera* × *V. berlanderi* rootstock exhibited arrested development of embryos at the heart-stage unless the culture medium was refreshed daily (Coutos-Thevenot *et al.*, 1992a), in which the authors suggested to be due to secretion of a > 10 kDa macromolecule (Coutos-Thevenot *et al.*, 1992b). However, a direct relationship between the extracellular material and embryo development was not shown.

Grape somatic embryos were shown to be able to survive severe dehydration, similar to that of seeds (Gray, 1987b, 1990a; Gray and Purohit, 1991b). During dehydration, the embryos became reduced in size and more discolored and brittle in consistency (Fig. 5A) (Gray, 1987b). Their water content equilibrated to 13% when stored at 70% RH and they resumed a normal appearance after rehydration. Genotypic differences in response were noted: culture lines that produced relatively well developed somatic embryos were most responsive (Gray, 1989). After 21 days of dehydrated storage, 34% of embryos from one grape genotype produced plants following imbibition (Table 2). Dehydrated embryos germinated to produce plants immediately after imbibition, whereas no plants were recovered from nondehy-

drated controls. Thus, dehydration served as a dormancy-breaking pretreatment.

The germination pattern of previously-dehydrated grape somatic embryos became remarkably altered (Gray 1987d, 1989). Fig. 5B compares germination patterns from embryos placed on various media. The untreated control embryo (Fig. 5B, embryo on left) remained white and produced only a shoot. Treatment with GA_3 resulted in limited germination (second embryo from left), whereas ABA caused profuse adventitious rooting and an epicotyl hook reaction (third from left). BA caused dramatic enlargement and adventitious rooting (embryo on right) and was the most reliable treatment for producing shoots. However, only previously-dehydrated somatic embryos were capable of germinating and growing in a synchronous pattern of root to shoot emergence resembling that of a seedling (Fig. 5B, embryo on bottom). This was in contrast to germination of nondehydrated embryos, which germinated abnormally. Although plants were obtained, notably with BA pretreatment, root and shoot emergence were not synchronous. Regardless of germination treatment utilized, all of over 300 plants that we have recovered from somatic embryos appear to be morphologically normal (ex. Fig. 5C).

An alternative method of plant recovery from somatic embryos was described by Vilaplana and Mullins (1989), who showed that shoot organogenesis from hypocotyls and cotyledons was a possible regeneration route.

2.6. *Growth in the field*

While it has not been difficult to establish somatic embryo-derived grapevines in the field, relatively little data exists. Krul and Mowbray (1984) compared somatic embryo-derived vines of *V. vinifera* "Seyval Blanc" with normally propagated vines and found that those from somatic embryos had darker leaves, reddish vs. green canes and cylindrical rather than conical fruit clusters. Furthermore, cuttings from regenerated vines rooted and grew more rapidly. The vines yielded 5 tons of fruit per acre three years after planting (Krul and Mowbray, 1984) and eventually 7.3 tons per acre (Krul, 1984). Although the somatic embryo-derived vines differed from existing, conventionally-propagated plantings, they corresponded closely to the original description of "Seyval Blanc". This prompted the authors to suggest that the basis for the observed differences was due to elimination of latent viruses during *in vitro* culture. Thus, in this instance, it appeared that regenerated vines were genetically stable. Such observations were further clarified by Goussard and Wiid (1992), who produced embryogenic cultures from vines infected with grape fanleaf virus in which cells and somatic embryos were shown to be infected. Immunosorbent electron microscopy and ELISA demonstrated that cultures were freed of viral infection by heat treatment at 35°C. Plants regenerated from the heat treated somatic embryos had normal, uninfected leaf morphology (Goussard and Wiid, 1992) and are now growing vigorously, producing exceptional fruit, with no virus symptoms or signs

of somaclonal variation (P.G. Goussard, personal communication). These observations of somatic embryo-derived grapevines and similar reports for other crops (i.e., Geier *et al.*, 1992), suggest that the process of somatic embryogenesis is remarkably resistant to genetic deviation, reinforcing the possibilities of controlled manipulation of the somatic genome.

2.7. *Applications of somatic embryogenesis*

Embryogenic culture technology is pivotal in applying many biotechnologies to grape improvement. A number of applications may rely on embryogenic cell cultures as described below.

Somatic hybridization. Success in somatic hybridization of grape will rest on our ability to regenerate plants from protoplasts. While there has been progress in isolation and culture methods, plant regeneration has yet to be obtained from grape protoplasts.

Leaf mesophyll tissue is the most commonly used source of protoplasts (Barbier and Bessis 1987, 1988, 1990; Lee *et al.*, 1989; Lee and Wetzstein, 1988; Nishimura *et al.*, 1984; Shimizu, 1985; Theodoropoulos and Roubelakis-Angelakis, 1990; Wright, 1985). Although isolation and culture protocols vary widely (Krul, 1988), most did not result in cell wall regeneration, cell division and sustained culture growth (Gray and Meredith, 1992). Therefore, it is difficult to determine the effect of specific protocols on the developmental events that lead to plant regeneration. The lack of progress in plant regeneration from grape protoplasts may be related to source tissue used. For example, success in plant regeneration from protoplasts of the Poaceae came only after development of specialized embryogenic suspension cultures that produced high yields of regenerative protoplasts (Gray, 1990b). A requirement for highly regenerative cell cultures in order to achieve plant regeneration from protoplasts is logical since, in practice, plant recovery rates from protoplasts are typically less than 10%. Currently, culture of *in vitro* leaves on regeneration medium designed to induce either embryogenesis or organogenesis yields very little plant regeneration when considering the large number of actual explant cells that are contained in a plated leaf. The chance of inducing a rare event such as plant regeneration from such leaves after protoplast isolation and culture seems remote indeed. With apparently adequate methodologies in place for isolation and culture, development of suitable culture lines is the next step toward achieving plant regeneration from protoplasts of grape. High yield of embryogenic protoplasts and regrowth back to cell cultures were obtained from the perennial-type cell cultures described above (Fig. 5D) (El-Gindy and Gray, unpublished data), however, plant regeneration has not yet been achieved. The current roadblock to plant regeneration must be removed before somatic hybridization, such as combining marginally sexually compatible *V. rotundifolia* and *V. vinifera*, can be accomplished.

Genetic transformation. Due the compelling applications for grapevine improvement, development of protocols to produce transgenic plants are underway worldwide. Transgenic cells and tissues were obtained by cocultivation with various *Agrobacterium* strains (Baribault *et al.*, 1989; Colby *et al.*, 1991; Gribaudo and Schubert, 1990; Guellec *et al.*, 1990); with the recovery of entire transgenic plants has been accomplished from organogenic cultures of *V. rupestris* (Mullins, 1990; Mullins *et al.*, 1990).

Baribault *et al.* (1990) produced shoots of *V. vinifera* "Cabernet Sauvignon" that contained both transformed and untransformed cells. Use of embryogenic cells or somatic embryos may be one approach to producing stably transformed *V. vinifera*. Embryogenic cells (Fig. 5E) and somatic embryos (Fig. 5F) of *V. vinifera* "Thompson Seedless" bombarded with DNA-coated tungsten particles were shown to transiently express the GUS gene (Gray and Songstad, unpublished data). Intact plants of transformed *V. vinifera* have been obtained by particle bombardment wounding, followed by *Agrobacterium* cocultivation (Scorza *et al.*, 1995). The "Thompson Seedless" cell line described herein was also transformed with this system. Transformed plants are GUS-positive and resistant to the antibiotic selection agent Kanamycin (Scorza *et al.*, in preparation). The coat protein-encoding gene for grape chrome mosaic nepovirus was inserted into tobacco (*Nicotiana tabacum* L.) and transgenic plants produced coat protein (Brault *et al.*, 1993). These plants were shown to be more resistant to inoculated infection than controls, providing evidence that a similar approach may be applicable in grape.

Synthetic seeds. Synthetic seeds are somatic embryos that are of commercial use in plant propagation (Gray, 1987a; Gray and Purohit, 1991a). While synthetic seeds of grape may not be useful for propagation, since adequate means are available and many vines are grafted, a significant application involves use of synthetic seeds for germplasm conservation (Gray and Compton, 1992). Because grape is self-incompatible, seeds cannot be used in germplasm conservation of distinct varieties or selections. Instead, germplasm conservation is accomplished by establishing plants in field germplasm repositories (orchards and vineyards), which are expensive and difficult to maintain over long time periods. For example, in the US, grape germplasm is preserved at the National Clonal Germplasm Repository (NCGR) at Davis, CA, and Geneva, NY. These locations house a combined total of 3900 grape accessions representing 41 and 34 species at each site, respectively. Two vines of each accession are maintained at a yearly cost of $ 25.00 per accession; however, the actual cost approaches $ 70.00 when administrative, research and evaluation cost are added (Gray and Compton, 1992). The problem of maintaining entire plants to accomplish clonal germplasm storage is so great as to have stifled or completely inhibited the collection of germplasm for many crops. These problems have prompted exploration of *in vitro* methods for conservation of clonal germplasm. Use of dehydrated quiescent somatic embryos, as described above, which could then be conserved in seed

repositories, represents a cost effective solution. Conservation via somatic embryogenesis would maintain germplasm purity and allow the amount of space required to house valuable collections to be reduced from several acres to just a few square feet of storage space. Such a conservation system would be preferable, since it would utilize existing seed storage facilities and techniques.

3. Conclusion

Grape is the worlds most valuable fruit crop due to its multiple uses and, as such, is the subject of considerable research emphasis. Somatic embryogenesis was first reported in 1976 for *Vitis vinifera* "Cabernet Sauvignon". Since that time, somatic embryogenesis has been obtained for an increasing number of additional species, hybrids and/or cultivars. Somatic embryo-derived grapevines appear to be normal in every respect. However, problems with initiating embryogenic cultures due to genotypic specificities persist and plant recovery rates from grape somatic embryos remain relatively low. Further technical development is compelling, since the embryogenic culture system is pivotal to applying a number of biotechnical procedures, including somatic hybridization, genetic transformation and synthetic seed technology, to grapevine improvement.

4. Acknowledgements

This is an updated and expanded version of pertinent portions of a previously published chapter (Gray and Meredith, 1992). Support was provided by the Program in Science and Technology Cooperation, Office of the Science Advisor, United States A.I.D. (grant # DHR-5600-G-00-0057-00). Florida Agricultural Experiment Station No. R-03386.

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SYNSEEDS

Applications of Synthetic Seeds to Crop Improvement

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Calgene, Inc.

Davis, California

CRC Press

Boca Raton Ann Arbor London Tokyo

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MAY 7 1996

COVER PHOTOGRAPH. Dr. Keith Walker watering newly planted field trial of alfalfa synthetic seeds in Yolo County, CA. Shown are row coverings of styrofoam cups and plastic tarps to impede desiccation of the synthetic seeds.

Library of Congress Cataloging-in-Publication Data

Synseeds : applications of synthetic seeds to crop improvement /
edited by Keith Redenbaugh.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-4906-0
1. Synthetic seeds. 2. Crop improvement. I. Redenbaugh, Keith.
SB118.46.S96 1992
631.5'21--dc20 92-22799
CIP

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International Standard Book Number 0-8493-4906-0

Library of Congress Card Number 92-22799

Printed in the United States of America 1 2 3 4 5 6 7 8 9 0

Printed on acid-free paper

Chapter 20

GRAPE SOMATIC EMBRYO DORMANCY AND QUIESCENCE: POTENTIAL OF DEHYDRATED SYNTHETIC SEEDS FOR GERMPLASM CONSERVATION

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0-8493-4906-0/93/$0.00 + $.50
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I. INTRODUCTION

Research in synthetic seed technology increasingly illustrates developmental similarities between zygotic embryos in seeds and somatic embryos (SEs) produced from cell cultures.[1] This is fortunate, because certain seed characteristics must be duplicated in SEs in order for them to be useful as synthetic seed.[2] For example, dried, quiescent SEs that mimic seed in their reversible arrested growth, were only recently documented for several genera.[3-6] Quiescent SEs can be stored in a dehydrated state and then germinated into plants after rehydration; this ability is crucial for a number of synthetic seed applications. Dormancy, a physiologically complex form of quiescence in which conditions or treatments in addition to rehydration (such as cold or heat) are required for germination, was also documented in SEs.[7,8] Such SEs may provide a convenient *in vitro* system for studies of dormancy.

The possibilities of utilizing SEs in the same manner as seeds offers intriguing applications in many areas of agriculture, both for crops that are currently seed-planted, as well as for those that are vegetatively propagated.[1] One application for vegetatively propagated crops involves use of synthetic seeds for germplasm conservation. Most vegetatively propagated crops, such as fruit and nuts, are self-incompatible, and do not produce genetically uniform seed. Therefore, seeds cannot be used in germplasm conservation of distinct varieties or selections. For such crops, germplasm conservation is accomplished by establishing plants in field germplasm repositories (orchards and vineyards); however, these are expensive and difficult to maintain over long time periods. The problem of maintaining entire plants to accomplish clonal germplasm storage is so great as to have stifled or completely inhibited the collection of germplasm for many crops. These problems have prompted exploration of *in vitro* methods for conservation of clonal germplasm.[9,10] One possible solution is to utilize quiescent SEs, which could then be conserved in seed repositories. Conservation via somatic embryogenesis would maintain germplasm purity and allow the amount of space required to house valuable collections to be reduced from several acres to just a few square feet of storage space.

In these early developmental stages of synthetic seed technology, it is necessary to refine model crop systems in order to test specific applications. Preferably, the models should have protocols for high-frequency somatic embryogenesis in place, and also have direct crop application in order to mitigate ultimate development costs.

Grape (*Vitis* spp.) is an excellent choice for testing the applications of synthetic seed for germplasm conservation, since embryogenic systems are available, certain aspects of synthetic seed technology already have been investigated, and substantial problems with clonal germplasm conservation exist. This chapter will present a case study dealing with the application of synthetic seed technology to germplasm conservation in grape. This will

include an overview of the crop, difficulties encountered in contemporary germplasm conservation, development of embryogenic culture systems, and the manipulation of quiescence and dormancy.

II. GRAPE

Grape is a deciduous temperate fruit crop of ancient origin. The genus *Vitis* is divided into two subgenera, *Euvitis* Planch., the bunch grape species that all contain 38 somatic chromosomes, and *Muscadinia* Planch., the muscadine grapes with 40 somatic chromosomes.[11,12] Species in each subgenus are interfertile, but are only partially fertile between subgenera.[13] Estimates of the number of *Euvitis* species vary widely, depending on taxonomic opinion, ranging from 28 to 43. These are separated into an American group of 18 to 28 species, an Asian group of 10 to 15 species, and a European or central Asian group of one species (*Vitis vinifera* L.).[11] *Muscadinia* contains three species; *Vitis munsoniana* Simpson and *Vitis rotundifolia* Michaux. are native to the southeastern U.S. and *Vitis popenoeii* Fennell is native to Central America. Natural hybridization occurs in regions where interfertile species overlap, leading to much genetic diversity and controversial taxonomy.

Several species of grape and their hybrids are currently cultivated.[11] Varieties of *V. vinifera* L., many of which originated in ancient times, account for the vast majority of world production. Hybrids between *Vitis labrusca* L., which is native to eastern North America, and *V. vinifera*, some with additional native species parentage, are in widespread production in the northeastern U.S.[14] Other hybrids between *V. vinifera* and southern U.S. grape species, form a unique series of Pierce's disease-resistant varieties that are adapted to humid climates.[15] Complex hybrids between *V. vinifera* and various native American species (French-American hybrids) are primarily used for wine production.[11,12,16,17] Muscadine grape, varieties of *V. rotundifolia* and *V. munsoniana*, are grown throughout the southeastern U.S. Additionally, clones from certain species and interspecific hybrids, selected from nature, are utilized as rootstocks or regional cultivars. For example, "Cynthiana" and "Norton", which produce premium red wine in the midwestern U.S., are considered to be selections of *Vitis aestivalis* Michx.[18] *Vitis champini* Planchon "Dog Ridge" and *Vitis rupestris* Scheele "St. George" were selected from the wild, and are regarded as valuable rootstocks used for nematode and phylloxera resistance, respectively.

Grape is very heterozygous and exhibits pronounced inbreeding depression.[11,12] Therefore, an individual genotype cannot be reproduced by seed, and outstanding selections must be propagated vegetatively. The life cycle is relatively long, with a juvenility period ranging from one to six years, depending on environmental conditions and management practices.[11] These characteristics combine to make manipulation of germplasm extremely difficult. Maintenance of germplasm with desirable characteristics, whether it arises

from nature or as a result of controlled hybridization, must be by vegetative means.

III. GERMPLASM CONSERVATION

Selection and breeding combined with monocultural practices have resulted in the narrowing of genetic diversity among clonally propagated crops such as grape. Therefore, it is important to preserve abandoned cultivars and land races that may possess resistance to both important and currently unknown insects, viruses, and diseases. The importance of such genotypes to plant breeders worldwide cannot be overemphasized. Clonally propagated crops are currently maintained in field gene banks (orchards, vineyards, or greenhouses) in order to preserve their genetic integrity. For example, in the U.S., grape germplasm is preserved at the National Clonal Germplasm Repository (NCGR) at Davis, CA and Geneva, NY. These locations house a combined total of 3900 grape accessions representing 41 and 34 species at each site, respectively.[19-20] At the Davis site, 2000 accessions are permanently planted on 4.9 ha. Vines are planted on a 2 × 4 m spacing, trained to a single stake, and spur-pruned. New material is obtained as cuttings or seed and propagated in the greenhouse before being established in the vineyard. Vines are continually indexed for virus and maintained as disease-free as possible.[20] Germplasm at the NCGR is available to breeders throughout the world. Since 1989, there have been approximately 3000 requests for grape accessions with about 75% from breeders in the U.S., and the remaining 25% from breeders in Germany, India, Japan, Pakistan, South Africa, Sri Lanka, and Russia.

Maintenance of clonal germplasm in field genebanks presents a number of problems. Collections can only be grown in areas with a suitable climate and are susceptible to natural disasters. Individual accessions require watering, pruning, fertilization, and spraying for insects and disease. Curators must repeatedly index and evaluate the material to identify threatening diseases and viruses. This results in high maintenance costs. For example, at the NCGR, two vines of each accession are maintained at a yearly cost of $25.00 per accession; however, the actual cost approaches $70.00 when administrative, research, and evaluation costs are added.[20,21]

The difficulty and high cost of storing accessions in field genebanks has stimulated exploration of alternative methods for clonal germplasm preservation. For example, researchers are currently investigating methods of storing grape pollen and dormant buds in liquid nitrogen. Grape pollen has been stored in liquid nitrogen for up to five years without a loss of viability,[22] but displayed reduced fertility as reflected by reduced berry and seed set.[23] Storing dormant buds in liquid nitrogen has been successful with *Vitis riparia*, but is currently less promising with other *Vitis* species.[24]

Another alternative to field genebanks is the storage of grape accessions as micropropagated cultures. Shoot tip cultures of eight *Vitis* genotypes have

been maintained at 9.5°C for 12 months without a change in ploidy.[25] Growth inhibitors such as Cycocel (CCC) can also be added to micropropagation medium to facilitate long-term storage. The addition of 500 to 1000 ppm CCC reduced internode length of micropropagated *Bacchus* and *Optima* shoot tip cultures by 30 to 40%, reduced the number of nodes per plantlet by 20 to 30%, and allowed the subculture interval to be increased from two to three months to five to seven months. Combining cold temperature storage at 8°C with 750 ppm CCC allowed *Bacchus* and *Optima* shoot tip cultures to be stored for ten months without a reduction in explant vigor.[26] Although a viable alternative to field genebanks, variation among genotypes in response, the considerable labor and space required to store micropropagated cultures compared to seed repositories, and the possibilities of chromosomal abnormalities, such as aneuploidy occurring due to cell division at low temperatures, may be limiting factors.

Somatic embryogenesis may lend itself to clonal germplasm storage in existing seed repositories, which are much more efficient than conventional clonal germplasm repositories. Accessions in seed repositories are stored in hermetically sealed, moisture-proof containers, and placed on movable racks in walk-in storage facilities, filed in boxes, or stored in chest freezers. The number of seeds stored per accession varies depending on the genetic homogeneity of the accession. The International Board for Plant Genetic Resources (IBPGR) Advisory Committee on Seed Storage recommends that 3000 to 4000 seeds be stored of genetically homogeneous accessions, whereas 4000 to 12,000 seeds are required for heterogeneous accessions.[27] Seeds in repositories are maintained as base collections and active collections. Base collections are maintained at temperatures below − 18°C and are seldom opened, whereas active collections are maintained at higher temperatures and are available upon request. A base and active collection is maintained for each accession. Somatic embryos and/or embryogenic cultures could be stored in a similar manner. For example, embryogenic cultures could be immersed in liquid nitrogen for long-term storage as base collections, while dehydrated and quiescent SEs could serve as convenient active collections. Such SEs would have the storage potential of seeds. Somatic embryos have been obtained from cryopreserved barley (*Hordeum vulgare* L.) callus,[28] suggesting that similar procedures could be developed for grape. Grape SEs already have been dried and stored in a manner similar to seeds, and plantlets have been obtained from rehydrated grape SEs.[3] Such a conservation system would be preferable, since it would utilize existing seed storage facilities and techniques. In the event of an accession request, SEs from active collections could be shipped, either before or after rehydration and germination, or germplasm could be supplied from base collections as cultures. However, improved methods of culture initiation and better dehydrated storage survival rates are necessary before this method can be employed as an alternative to field genebanks.

IV. SOMATIC EMBRYOGENESIS

Grape somatic embryogenesis was first reported by Mullins and Srinivasan[29] in 1976, who cultured unfertilized ovules of *V. vinifera* "Cabernet Sauvignon" in liquid medium that contained naphthoxy acetic acid (NOA) and benzyladenine (BA). Proliferating nucellus-derived callus was extruded through the ovular micropyle, and eventually gave rise to SEs. Somatic embryos germinated into plants when transferred to agar-solidified medium that contained gibberellic acid (GA_3) and 2-isopentenyladenine (2-iP). Krul and Worley[30] documented somatic embryogenesis from calli derived from leaf, petiole, and stem segments of the French-American hybrid "Seyval" cultured on solidified medium containing 2,4-dichlorophenoxy acetic acid (2,4-D). Embryos developed after transfer of callus to medium containing α-naphthalene acetic acid (NAA) and BA. Embryos germinated into plants after transfer to medium devoid of growth regulators. The original embryogenic line could be maintained indefinitely through secondary embryogenesis on growth regulator-free medium. All successive reports concerning the induction of embryogenic cultures from grape have followed protocols similar to these early reports.

To date, somatic embryogenesis has been obtained from only three grape species: *Vitis longii* Prince, V. *rupestris* Scheele, or *V. vinifera*. All hybrids that have produced embryogenic cultures are composed of at least one of these.[31] Additionally, genotypic specificities are noted within species. For example, only nine cultivars of *V. vinifera* have produced SEs. Most successful reports relied on floral tissues, particularly anthers or ovules (with the nucellus documented to be the source of embryogenic cells in the latter); however, leaves, shoot tips, and zygotic embryos have also been utilized.[30,32-34] Explant developmental stage as well as preconditioning also may be important in obtaining embryogenic cultures.

Once initiated, embryogenic cultures are maintained either by continual transfer on medium containing auxin, with or without cytokinins, or subculture on medium devoid of growth regulators. Somatic embryos develop either from callus material or directly from previously-formed embryos. Somatic embryos appear to grow from single embryogenic cells via a suspensor.[30,32] Starch is not present in appreciable amounts in either suspensors or somatic embryos, and lipid, which is abundant, appears to be the predominant storage compound.[32] Somatic embryos pass through recognizable developmental stages, possess normal epidermal, cortical, and vascular tissues, and tend to be opaque white in color from the early torpedo stage to germination. Somatic embryos that are anatomically and morphologically faithful to all zygotic embryo stages are commonplace; however, a number of developmental abnormalities often occur. The most common abnormalities include the development of more than two, often fused, cotyledons and SEs that are larger than zygotic embryos.[32] These abnormalities are not likely to be related to genetic changes, since plants of normal appearance have been recovered from such embryos.

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Although plant recovery from grape SEs has been possible from the first report, (for example, see Reference 29), specific germination requirements have varied widely. Germination and plant recovery from SEs are often improved by pretreatments with cold, auxins, cytokinins, or GA_3.[3,7,32,35] The positive effect of such pretreatments on germination led researchers to investigate the possibility that dormancy occurred in grape SEs.

V. DORMANCY

To better understand the germination requirements exhibited by grape SEs, it is useful to consider zygotic embryos in grape seed, which exhibit a type of dormancy that is alleviated by cold stratification. Germination occurs after approximately four weeks of treatment at 4°C.[36] Grape SEs mimic seed in that they germinate better following cold treatment, providing evidence to suggest that they are also dormant.[32] Studies of abscisic acid (ABA) concentration changes in grape embryogenic cultures showed a rapid increase during embryo development, which reached a peak at maturation.[8] Cold stratification of SEs resulted in a rapid decrease in ABA. Exogenously supplied ABA inhibited SE germination.[7] Because ABA is implicated as a controlling factor of dormancy in many types of seeds, (for example, see References 37 to 39), it is plausible to consider that ABA also functions similarly in grape SEs. In contrast to ABA, GA_3 supplied exogenously induced grape SE germination, and the concentration of endogenous GA-like compounds increased during cold stratification.[40] This suggests a simple endogenous control of embryogenesis and germination for GA. In contrast, ABA inhibits precocious germination, and thus promotes normal development. Embryo dormancy appears to be a developmental mechanism that promotes full maturation, and may allow germination to be timed to the season that is most amenable to successful plant establishment (in the case of grape, spring). The close resemblance of grape somatic- and zygotic embryo dormancy suggests that dormancy also exists in other embryogenic culture systems with corresponding zygotic embryo dormancy, especially when the SEs become morphologically well-developed, but still fail to germinate.

VI. INDUCTION OF QUIESCENCE

Embryos that develop in orthodox seed, such as grape, begin a resting period during late stages of maturation, that also coincides with gradual dehydration. This dehydrated resting phase, which is missing in all conventional embryogenic culture systems, is the major characteristic that allows seed to be conveniently stored.[2] The ability of seed to withstand dehydration is critical to attainment of the resting phase. Seed embryos must be at the "desiccation tolerant stage" in order to survive dehydration.[2,41-43] Prior to dehydration, metabolism is oriented toward accumulation of storage com-

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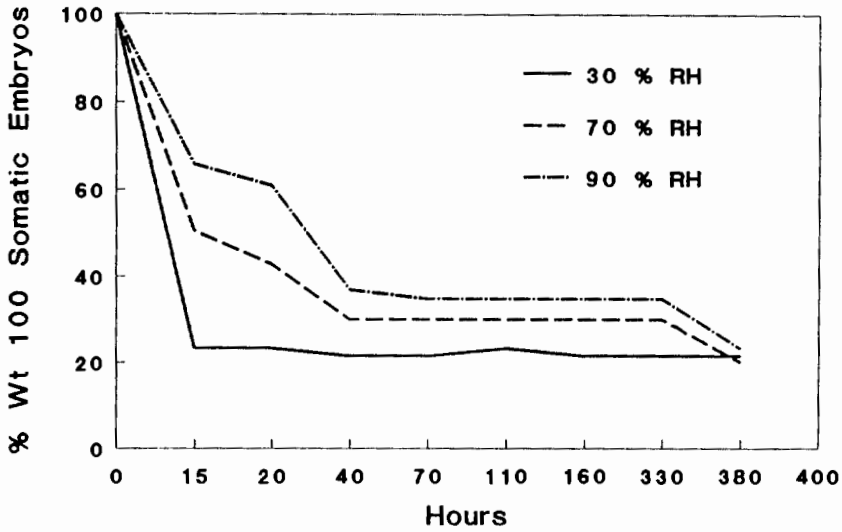

FIGURE 1. Weight loss by grape SEs maintained at 25°C under 30, 70, or 90% RH. Note that weight loss is greater and more rapid at lower RHs. Weight loss after 330 h was due to additional drying at 60°C to remove remaining water. This demonstrates that RH can be used to modulate drying rate and control equilibrated water content in SEs.

pounds, whereas after rehydration, storage reserves are consumed for germination. These observations suggest that dehydration followed by rehydration functions to switch the mode of development from that of storage synthesis to catabolism of storage material.[44] Therefore, acquisition of desiccation tolerance appears to be central to normal embryo development. Dehydration may cause a basic switch in physiology, such that pathways favoring embryo development are replaced by those that lead to plant development. Poor germination of SEs may be due to the absence of these complex developmental mechanisms. Induction of desiccation tolerance, followed by dehydration and quiescence, may prompt SEs to function in a manner more like that of seed embryos.

Somatic embryo dehydration can be finely regulated by utilizing a controlled relative humidity (RH) environment, as illustrated by Figure 1, which shows the weight loss over time by groups of 100 grape SEs when exposed to 30, 70, or 90% RH air. Embryos in 30% relative humidity dehydrated most rapidly and lost the greatest percentage of weight, whereas the reverse was true for the 90% level. The embryos were placed in a 60°C oven after 330 h to remove additional unbound water, resulting in a sharp drop in weight for the 70 and 90% treatments, such that all three became equivalent. By comparing fresh weights with those obtained at equilibrium at each RH with dry weights, we calculated that nondehydrated embryos contained 75% water, whereas those maintained at 30, 70, and 90% RH until weight stabilization

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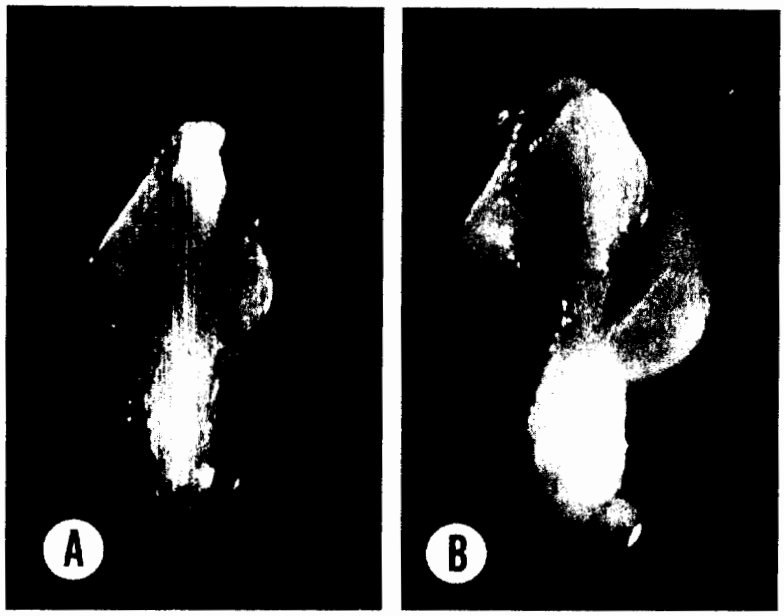

FIGURE 2. Dehydrated (A) and rehydrated (B) grape SEs. Dehydrated embryos are discolored and reduced in size, but enlarge and assume a normal appearance after rehydration.

occurred, contained 22, 30, and 35% water, respectively. This demonstrates that distinct SE water contents can be produced and maintained with this protocol.

During dehydration, grape SEs became discolored and greatly reduced in size (Figure 2A), but resumed a normal appearance upon rehydration (Figure 2B).[3] Genotypic differences in response were noted; culture lines that produced relatively well-developed SEs were most responsive.[7] After 21 days of dehydrated storage, 34% of the embryos from one grape genotype produced plants following imbibition (Table 1). Dehydrated embryos germinated to produce plants immediately after imbibition, whereas no plants were recovered from nondehydrated controls. Prior to our investigations of dehydration, the best treatment for inducing germination and producing plants from grape SEs was to break dormancy by treatment with BA. This study demonstrated that higher germination percentages were possible through dehydration. Since dehydrated SEs grape do not require typical dormancy breaking pretreatments and germinate directly after imbibition, they can be considered to be quiescent, not dormant. Alternatively, dehydration may be viewed as a dormancy-breaking treatment. This latter conclusion is supported by studies of somatic embryogenesis in red oak (*Quercus rubra* L.) and soybean.[45,46] Somatic embryos of red oak germinated and produced shoots only after a period of dehydration

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TABLE 1
Comparison of Dehydration and Benzyladenine for Inducing Germination in Grape Somatic Embryos

Treatment	Percent germination response[a]			
	Hypocotyl	Root	Cotyledon	Shoot
Dehydration	77	68	65	34
0.5 μ*M* BA	100	92	98	12
Control	76	88	36	0

Note: Well-developed embryos were either (1) dehydrated for 21 days at 70% RH and 27°C, (2) placed directly on medium with benzyladenine (BA), or (3) placed on basal medium (control).

[a] Germination response was based upon either enlargement and greening of hypocotyls and cotyledons or emergence of roots or shoots.

From Gray, D. J., In Vitro *Cell Dev. Biol.*, 25, 1173, 1989. With permission.

or treatment with osmotically active sugars. With soybean, only a few SEs from several test genotypes germinated without dehydration pretreatments, but up to 100% germinated after dehydration.

One unexpected result of our dehydration experimentation was that the germination pattern of grape SEs could be strikingly manipulated.[7] Only SEs that had been dehydrated were capable of germinating in a pattern reminiscent of seedlings such that shoot and root emergence occurred synchronously (Figure 3). This was in contrast to germination of nondehydrated control embryos with and without treatment with dormancy-breaking growth regulators. These embryos germinated abnormally such that embryonic organs often became enlarged. Although plants were occasionally obtained, notably with BA pretreatment, root and shoot emergence was not synchronous.

VII. CONCLUSION

Synthetic seed technology has compelling applications for a number of crops. However, induction of somatic embryogenesis remains limited to a few responsive genotypes, and satisfactory storage periods have not yet been obtained. These factors must be improved before synthetic seed will be utilized for germplasm conservation.

FIGURE 3. Comparison of germination treatments for grape SEs. From top left: control treatment; 1 μ*M* GA; 1 μ*M* ABA; 1 μ*M* BA. Bottom: dehydration at 70% RH for 21 days. (From Gray, D. J., In Vitro *Cell Dev. Biol.*, 25, 1173, 1989. With permission.)

ACKNOWLEDGMENTS

Partial support for this project was provided by the Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development under Grant No. DHR-5600-G-00-0057-00, and by the International Board for Plant Genetic Resources (IBPGR), as part of the IBPGR Research Program. This is Florida Agricultural Experiment Station Journal Series No. R-01895.

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SYNSEEDS

Applications of Synthetic Seeds to Crop Improvement

Edited by

Keith Redenbaugh, Ph.D.
Calgene, Inc.
Davis, California

CRC Press
Boca Raton Ann Arbor London Tokyo

REC'D IN R&D/R
MAY 7 1996

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COVER PHOTOGRAPH. Dr. Keith Walker watering newly planted field trial of alfalfa synthetic seeds in Yolo County, CA. Shown are row coverings of styrofoam cups and plastic tarps to impede desiccation of the synthetic seeds.

Library of Congress Cataloging-in-Publication Data

Synseeds : applications of synthetic seeds to crop improvement /
edited by Keith Redenbaugh.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-4906-0
1. Synthetic seeds. 2. Crop improvement. I. Redenbaugh, Keith.
SB118.46.S96 1992
631.5'21--dc20 92-22799
CIP

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International Standard Book Number 0-8493-4906-0

Library of Congress Card Number 92-22799

Printed in the United States of America 1 2 3 4 5 6 7 8 9 0

Printed on acid-free paper

Chapter 19

LIQUID SUSPENSION CULTURE PRODUCTION OF ORCHARDGRASS SOMATIC EMBRYOS AND THEIR POTENTIAL FOR THE BREEDING OF IMPROVED VARIETIES

Dennis J. Gray, Robert N. Trigiano, and Bob V. Conger

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0-8493-4906-0/93/$0.00 + $.50
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INTRODUCTION

A number of applications for synthetic seed technology have been envisioned, including applying it to field planting agronomic crops.[1,2,3] However, the cost of synthetic seed for most seed-propagated crops must be very low to constitute a useful alternative.[3] Automated production of the synthetic seeds will be necessary to reach such an acceptable cost range.[4] Furthermore, synthetic seed produced for field planting must closely resemble normal seed in form and function in order to be compatible with conventional farming practices. Therefore, a functional synthetic seed for this type of application will consist of a dehydrated, quiescent somatic embryo (SE), protected by an encapsulation.[3,5]

One advantage of utilizing somatic embryos (SEs) for plant propagation is the anticipated suitability for rapid and cost effective production of large numbers of clonal plants in automated culture systems.[6] Liquid medium-based culture systems, generally termed "bioreactors," are the best prospects for automated production, since they can be easily manipulated by simple pumping, sieving and/or agitation of medium or tissue.[7-10] All of these steps can be automated with existing technology.

Somatic embryos develop with a complete shoot/root axis. Consequently, the production of propagules capable of germination into complete plants appears to be readily possible in such systems. Conceivably, a cell culture could be manipulated in an automated system to produce embryos, which then could be isolated, encapsulated, and dried.[11] However, there are few examples where SE development and maturation occur directly in liquid culture. Although a number of automated or semi-automated systems have been described, difficulties have been encountered in regulating and synchronizing embryo development to the degree necessary for normal morphological and physiological maturation.[3]

Overcoming the obstacle imposed by incomplete and asynchronous SE development has been more difficult than originally expected, and this has stifled refinement of automated synthetic seed production systems. Use of liquid culture systems that currently exhibit superior embryogenic capacity as models appears to be the best approach to alleviating this obstacle and demonstrating the utility of synthetic seed technology for other systems. In order to minimize development costs for commercial application, we feel that synthetic seed technology should also bear an application for the specific model species chosen.

In this chapter, we evaluate potential applications of synthetic seed technology for orchardgrass (*Dactylis glomerata* L.) improvement and propagation. A particularly well-refined suspension culture system as well as SE dehydration has been described for this species.[12-15] This chapter will begin with an overview of the species and a rationale for use of synthetic seed technology for this and similar crops. Production of SEs in liquid suspension

cultures as well as progress toward inducing reversible quiescence through dehydration will be reviewed in light of reports for other species.

II. ORCHARDGRASS

Orchardgrass is a perennial, seed-propagated, temperate forage grass. It is native to Europe, but has been grown in North America for more than 200 years.[16] Orchardgrass grows and spreads by the production of tillers. In contrast to grasses like bermudagrass (*Cynodon dactylon* L.), which produce stolons that can be cut into pieces and planted for stand establishment, tillering grasses cannot be efficiently clonally propagated due to lack of suitable vegetative propagules. Therefore, orchardgrass pastures must be established from seed. Since orchardgrass is self-incompatible and naturally outcrossing, it is essentially impossible to maintain the genotype and phenotype of outstanding individuals. Conventional breeding methods primarily utilize a synthetic approach in which many replicates of a few to several clones previously selected for superior agronomic characters, synchrony of flowering date, combining ability, etc., are allowed to intercross at random. These procedures require long periods (15 to 20 years), and the progress in synthesizing new cultivars, which are significantly superior to those currently grown, has been slow.[17] For example, one of the most widely grown cultivars in the U.S. is "Potomac", which was released in 1954 and took 19 years to develop.[16,18]

Orchardgrass is generally regarded to be an autotetraploid (4x = 28), based on tetrasomic inheritance of several characters and by observance of mean quadrivalent frequencies of between 3 and 5% per cell.[19,20] Further evidence for autotetraploidy has been provided from recent studies of chloroplast DNA variation.[21] Gametophytic incompatibility has been interpreted as a tetrasomic two-locus "grass system" with one SF allele at one compatibility locus.[22] The complexity imposed on breeding due to this genetic system, combined with few phenotypic traits that can serve as genetic markers, makes orchardgrass difficult to improve by controlled hybridization.

As mentioned above, progress in making significant gains through conventional synthetic breeding procedures has been slow. Therefore, grass breeders have long been interested in alternate and supplemental approaches for cultivar development. Synthetic methods provide little opportunity to utilize heterozygosity, let alone maximize it. Heterozygosity is important in maximizing phenotypic performance of polysomic polyploids. To accomplish this, genotypes must be developed and selected which have favorable tri- and tetra-allelic interactions.[23] Plant breeding involves both intragenomic and intergenomic repatterning.[24] In breeding of polyploids, separation of these two phases is not easy, and breeding progress is often slow. This has certainly been true for orchardgrass. Chase proposed the term "analytic breeding" for resolving a polyploid into its constituent parts, the basic genomes.[25] It is then possible to treat the problems of intra- and intergenomic breeding separately. The three-phase process involves: (1) reduction of a polyploid to its basic

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genomes (analysis), (2) intensive breeding and selection at the diploid sporophyte level, and (3) resynthesis and testing of the polyploid form. Analytic breeding schemes, or modifications thereof, have already been successfully used in other autotetraploid crops, i.e., in alfalfa and potato.[23,26]

An essential requirement of analytic breeding is reduction of the genome to the gametic chromosome level. In orchardgrass, this would be from 4x to 2x. In potato and alfalfa, reduction was accomplished by parthenogenesis or maternal haploidy. In orchardgrass this may be accomplished by anther or microspore culture. Breeding and selection would be done at the diploid (2x) level. Selected phenotypes could be hybridized at both the 2x and 4x (after scaling-up) levels. Increased heterozygosity would be expected in progeny from double crosses and double-double crosses, as was shown in alfalfa.[23]

The use of synthetic seeds would be advantageous for propagation at various stages of an analytic breeding scheme for orchardgrass. This would include the 2x derivatives from 4x parents, hybrids between the 2x derivatives, and plants scaled-back up to the 4x level to be used in double- and double-double crosses.

III. EMBRYOGENIC SUSPENSION CULTURE

Many early attempts at suspension culture of grasses and cereals resulted in development of only roots or nonmorphogenic material. This led to a high degree of pessimism regarding the possibilities of developing regenerative cultures in these species, as evidenced in published reports.[27,28] However, improvements in methodology led to development of cultures capable of sustained production of embryogenic tissue. In addition to orchardgrass, embryogenic suspension cultures have been reported for annual ryegrass (*Lolium perenne* L.),[29] corn (*Zea mays* L.),[30,31] guinea grass (*Panicum maximum* Jacq.),[32] Italian ryegrass (*Lolium multiflorum* Lam.),[29,33] napier grass (*Pennisetum purpureum* Schum.),[34] pearl millet (*Pennisetum americanum* (L.) Schum.),[35] rice (*Oryza sativa* L.),[36] sugarcane (*Saccharum officinarum* L.),[37] and tall fescue (*Festuca* arundinacea Schreb.).[29]

A key factor in initiating and establishing embryogenic suspension cultures is the selection of inoculum with regenerative potential. Embryogenic callus, SEs, and zygotic embryos have all been used successfully as inoculum.[12,33] Friable-type callus is preferable to the compact, nonfriable-type callus or organized tissue, since it tends to disperse readily into liquid medium.[30] Both embryogenic callus and SEs were used to initiate embryogenic suspension cultures of orchardgrass.[6,12,15] The culture initiation phase is often slow, requiring procedures not needed in culture maintenance. For example, in a described protocol for corn, cell suspensions could not be divided during the first month of culture.[30] Instead, the medium was replaced weekly during the first month, and, then, the medium volume was doubled weekly for the next two months. Afterward, the culture could be maintained and increased by diluting one volume of culture with five volumes of fresh medium.

A different protocol was used to establish embryogenic suspension cultures of orchardgrass.[12] Small pieces of embryogenic callus or isolated SEs were placed into 24-multiwell plates (capacity 3.6 ml per well) filled with 1 ml of SH-30 medium (Schenk and Hildebrandt medium[38] amended with 30 μM of the synthetic auxin, dicamba). The plates were agitated on a rotary shaker, and 1 ml of fresh medium was added to each well after 14 days. Under these conditions, the scutellar epidermis of SEs proliferated to form a friable mass of cells within the medium. After an additional 14 days, cultures from several wells were combined and added to a 10 ml flask containing 5 ml of fresh medium. After 14 days, the cultures were placed in a 125 ml flask and progressively transferred to 10, 20, and 30 ml volumes of fresh medium every 14 days. Resulting cultures were maintained by replacing spent medium with fresh medium every 7 days and dividing the cultures in half every 14 days.

Among species of the Poaceae, orchardgrass is unique in that SEs capable of germination into plants develop from long-term cultures directly in liquid medium.[6,12,15] Embryogenic suspension cultures of the other poaceous species mentioned previously do not produce morphologically mature embryos during the liquid culture phase. Rather, cells isolated from such cultures must be first grown into a callus on solidified medium for embryogenesis to occur (for example, see References 30, 32, 37). Embryo production directly in liquid medium is a prerequisite to automated synthetic seed production.

In orchardgrass, embryogenesis was controlled by the presence of organic nitrogen in the form of either casein hydrolysate or various amino acids.[6,15] The effect of casein hydrolysate on embryogenesis was dramatic. Although capable of sustained growth, cell suspensions maintained without casein hydrolysate did not produce recognizable SEs. Such cultures proliferated as cell masses that frequently bore root primordia. When removed from the suspension and placed on solid medium, these cell masses developed into a callus that produced SEs.

The addition of casein hydrolysate to established cultures stimulated the rapid development of SEs, which matured to a germinable stage directly in the liquid culture medium.[6,12,15,39] Somatic embryos frequently accumulated en masse at the surface of the medium along the edge of the culture vessel, and were first noticeable by their globular, opaque-white appearance. Although several embryological stages in addition to abnormal embryos were present in the cultures at any given time, morphologically normal SEs were routinely isolated. Embryogenesis was reliably initiated and halted by addition or removal of casein hydrolysate, respectively. The embryogenic response was found to be related to both casein hydrolysate and dicamba concentration; a combination of 3 to 4 g/l casein hydrolysate and 40 μM dicamba produced the optimum effect (Figure 1). Under these conditions, over 2300 recognizable SEs were formed from an initial 1 ml of inoculum in 30 ml of medium after four weeks of growth. Somatic embryos were reliably isolated on a 180 μm mesh screen from cultures that were previously passed through a 1050 μm

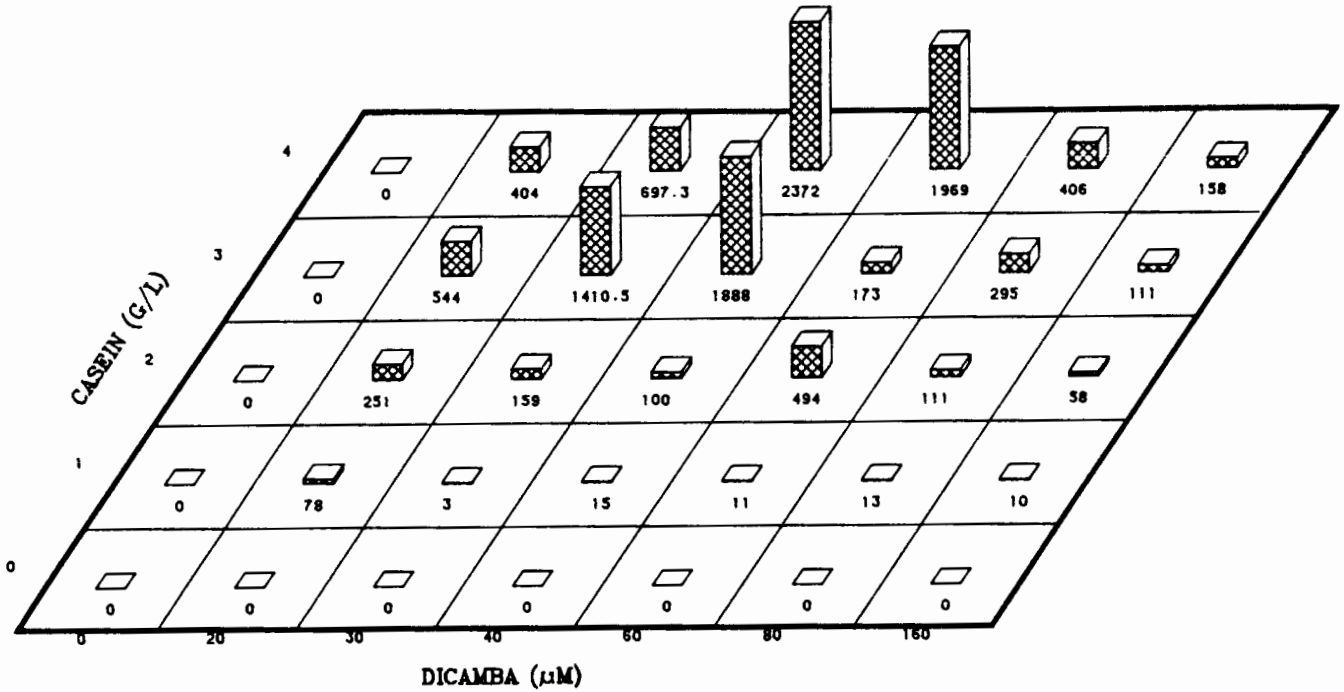

FIGURE 1. Effects of dicamba and casein hydrolysate on SE number in suspension cultures of orchardgrass. (From Gray, D. J. and Conger, B. V., *Plant Cell Tissue Org. Cult.*, 4, 1232, 1925. Reprinted by permission of Kluwer Academic Publishers.)

screen (Figure 2, top). Such embryos germinated into plants when placed on solidified or liquid, growth regulator-free medium. Germination into plants was also obtained by placing SEs directly into sterile distilled water (Figure 2, bottom). Apparently the SEs possessed adequate storage reserves to survive under these conditions.

An analysis of the casein hydrolysate (acid hydrolysate type II, Sigma Chemical Co., St. Louis, MO) utilized in this study showed that it contained 13.3% total nitrogen, 73% of which was in the form of 18 different amino acids,[55] suggesting that organic and/or inorganic nitrogen was responsible for the casein hydrolysate effect. Consequently, the effects of nitrogenous compounds, including specific amino acids or combinations thereof, on orchardgrass suspension cultures were evaluated.

In experiments that evaluated the effects of amino acids and ammonium ion on growth and somatic embryogenesis, cultures were first incubated with casein hydrolysate to assess embryogenic potential.[39,40] Cultures were then transferred to basal medium lacking casein hydrolysate and transferred at weekly intervals for five weeks. At the end of this period, lack of visible embryogenesis was verified, and the cultures used to inoculate experimental medium.

Specific compounds added to basal medium to stimulate somatic embryogenesis profoundly influenced growth characteristics of the culture as well as quantity, and more importantly, quality of SEs. Cultures grown in medium containing casein hydrolysate consisted of individual cells, large cell aggregates, proembryogenic masses in which embryos were embedded, and individual embryos.[15,41] Embryos formed under this culture regime displayed callusing of epidermal cells, and as many as 40% of the embryos had germinated precociously.[15,41,42] In other experiments, when cells were initially cultured in SH-30 medium amended with either 12 or 25 m*M* ammonium ion, supplied as $(NH_4)_2SO_4$, embryogenesis and embryo development were unregulated; embryos callused extensively and produced secondary and tertiary embryos from scutella and coleorhizae of primary embryos.[40] The majority of embryos that developed in ammonium-supplemented medium did not form plantlets when transferred to germination medium. These media, while promoting the production of large numbers of SEs, would not be suitable for use in large scale bioreactors because of lack of embryo singulation and control of embryo development and germination.

A logical progression from using amino acid-rich casein hydrolysate to regulate embryogenesis was to examine the influence of individual and combinations of amino acids on embryogenesis and development of SEs. Previous reports indicated that proline improved the frequency of embryogenic callus initiation and increased embryogenesis in cultures of corn on semisolid medium.[43] Although proline supports growth equivalent to that of casein hydrolysate in suspension cultures of orchardgrass, it did not stimulate embryogenesis. Serine and other individual amino acids inhibited growth as well as embryogenesis, when used as the sole addendum to SH-30 medium.[15,40] How-

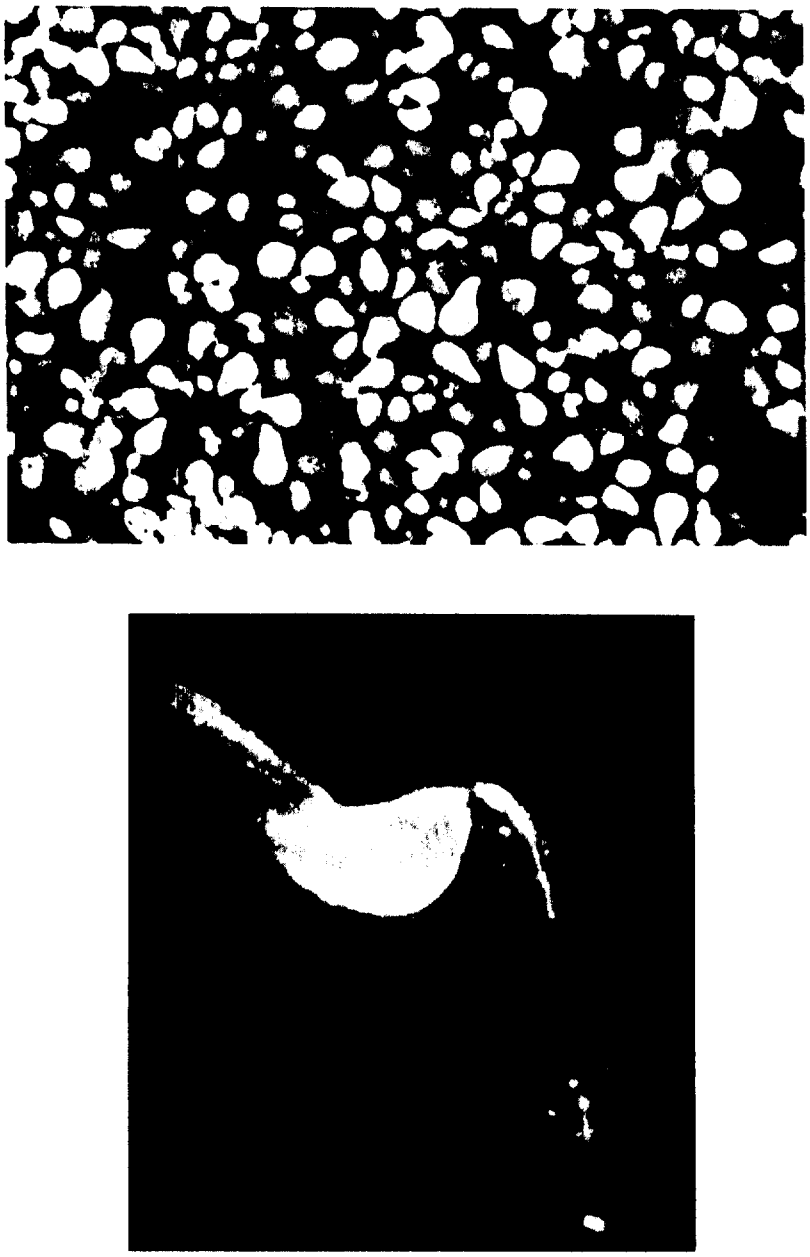

FIGURE 2. Orchardgrass SEs derived from suspension cultures containing 30 μM dicamba and 3 g/l casein hydrolysate. (Top) Embryos collected on a 180 μm mesh sieve; (Bottom) SE germinated in distilled water.

ever, the inhibitory effects of individual amino acids on growth were somewhat alleviated, and somatic embryogenesis was promoted when equimolar concentrations (12.5 m*M*) of proline and either serine or threonine were added to the culture medium.[15,40] Such suspensions were composed primarily of small cell aggregates and individual cells; in this regard, they resembled suspension cultures reported for corn.[31] Embryos produced in medium supplemented with these amino acids formed singly, did not proliferate cells from the epidermis, and exhibited little precocious germination. Although fewer embryos were often obtained when compared with cultures grown with casein hydrolysate, these closely resembled the embryos formed in medium containing abscisic acid (ABA), and over 80% germinated when placed on basal medium without dicamba.[40,42]

As a prerequisite to mass production of popagules employing large volume bioreactors, a long-lived and reliable culture system that requires minimal cultural and mechanical manipulation must be developed. Orchardgrass suspension cultures would seem to be very amenable to this technology in that they retain embryogenic potential over extended culture periods and produce fully developed embryos capable of germination in a single medium. Furthermore, embryos are produced singly, thus eliminating the need for mechanical separation from callus masses, and little precocious germination occurs.

IV. DEHYDRATION AND INDUCTION OF QUIESCENCE

When considering synthetic seed applications, an important developmental difference between zygotic and SEs is that SEs lack a quiescent resting phase.[11] Most zygotic embryos in seeds begin a resting period during maturation, which is the major factor allowing seed storage.[2] Basic developmental mechanisms that induce or allow the resting phase differ among species. Two types of arrested growth, "quiescent" and "dormant", are generally distinguished.[44] Quiescence is a resting phase that can be reversed solely by the addition of water, whereas dormancy requires factors in addition to water, such as cold or heat treatments. Quiescent seeds are less difficult to manipulate and, therefore, more convenient than dormant seeds. Seeds of the majority of crop plants are of the quiescent type.

Dehydration may be a controlling factor in onset and regulation of the resting phase. In seeds, maturation ripening occurs concomitantly with dehydration. Metabolism decreases during dehydration, culminating in onset of the resting phase. Metabolism increases after rehydration and germination occur.[45] Seeds must reach a desiccation-tolerant stage in order to withstand dehydration.[11,46,47] Metabolism is oriented toward accumulation of storage compounds prior to dehydration, whereas storage reserves are consumed after rehydration as a consequence of germination. Kermode et al.[45] suggested that dehydration followed by rehydration functioned to switch the mode of de-

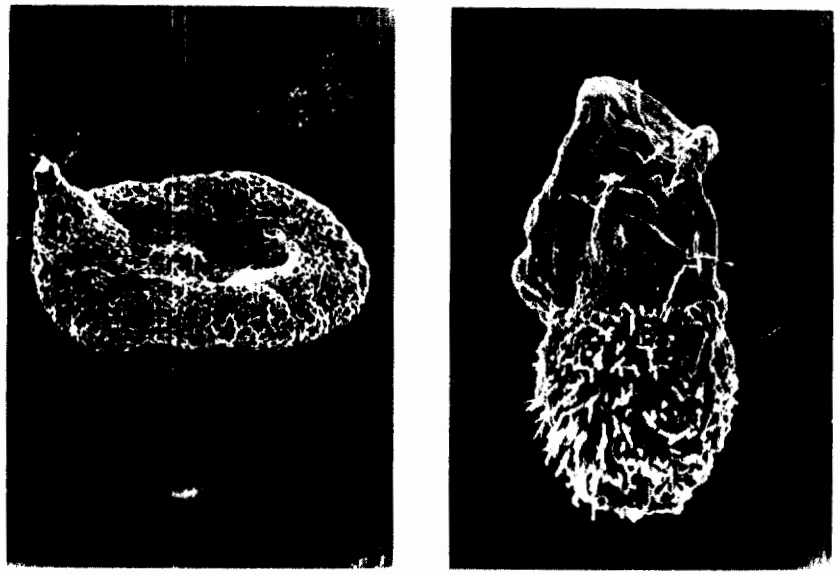

FIGURE 3. Dehydration of orchardgrass SEs. (Left) SEM of embryo after three weeks of dehydration; (Right) SEM of rehydrated SE. Note root hairs emerging from coleorhiza region denoting that the embryo had survived dehydration and germinated. (From Gray, D. J., Conger, B. V., and Songstad, D. D., In Vitro *Cell Dev. Biol.*, 23, 29, 1987. With permission.)

velopment from one of storage synthesis to the reverse, catabolism of storage material. Therefore, dehydration appears to be an important occurrence in embryo development.

Lack of a resting phase in SEs is a major obstacle to their use as synthetic seed.[2] Somatic embryos with reversible, arrested growth will be needed in order to mimic seed storage and handling characteristics. Quiescence and dormancy have only recently been recognized in SEs, and it is possible that dormancy occurs naturally in culture systems where plants cannot be obtained from well-developed SEs (for example, see Reference 48). This suggests that traditional methods for manipulating quiescence in seeds (for example, see Reference 49) may be applicable for SEs.

A. QUIESCENCE IN ORCHARDGRASS

Use of dehydration to prompt SEs to enter a resting phase is logical, since dehydration and rehydration can result in arrest and resumption of growth in seeds, respectively. Recovery of viable plants from dehydrated SEs was reported first with orchardgrass.[11,13,14] The SEs dehydrated under 70% relative humidity (RH) air became discolored and decreased in size (Figure 3, left). Embryo water content dropped from 83 to 13% within 24 h, (Figure 4), and was maintained over experimental storage periods of 7 to 21 days. Somatic embryos were rehydrated on solid medium, during which they swelled rapidly,

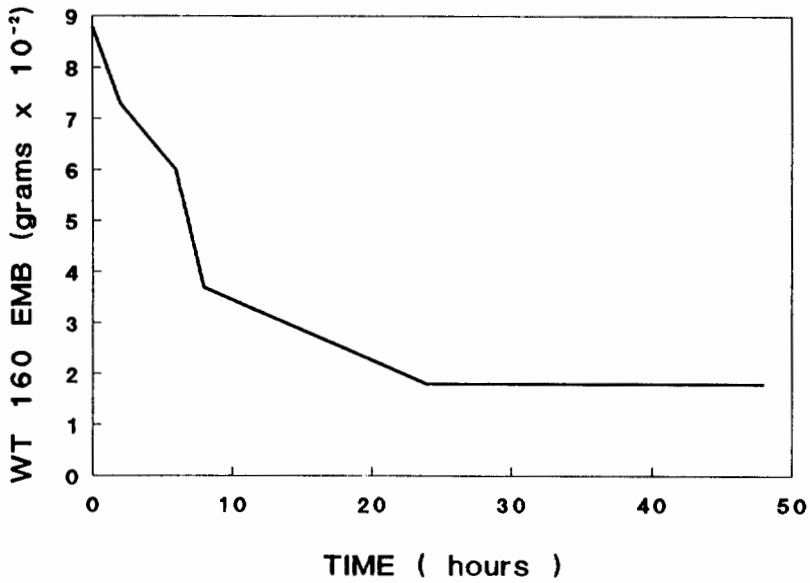

FIGURE 4. Weight loss by orchardgrass SEs during dehydration (From Gray, D. J., Conger, B. V., and Songstad, D. D., In Vitro *Cell Dev. Biol.*, 23, 29, 1987. With permission.)

regained their normal white coloration, and germinated (Figure 3, right). Only well-developed (possessing a visible scutellum and coleoptile), white, opaque SEs were responsive. Such SEs were structurally mature and contained starch and lipid storage compounds. When these embryos were stored in a dehydrated state for 21 days, 4% germinated and produced plants of normal appearance after imbibition (Table 1; Figure 5). A water content of 13% is similar to that utilized for maintenance of seed viability during prolonged seed storage. The ability of SEs to survive a reversible arrested growth phase, induced by similar water contents, demonstrates the occurrence of quiescence.

B. QUIESCENCE IN OTHER CROPS

Induction of quiescence in SEs with plant recovery was subsequently obtained in several other species. Grape SEs were dehydrated by the method described for orchardgrass, and quiescence was induced.[50] (See Chapter 20 of this volume). Quiescence also was observed in SEs of corn and soybean,[56] and it was successfully induced in SEs of alfalfa.[51] Approximately 60% of high quality alfalfa SEs produced plants after one year of dried storage.[47] Quiescence in alfalfa was attributed to a pulse of ABA, applied to 14-day-old SEs, which induced a state of desiccation tolerance. Such pulse-timed applications of ABA in conjunction with controlled drying approximates certain aspects of the developing seed environment. Documentation of desiccation tolerance and quiescence in SEs demonstrates that they are capable of entering complex developmental pathways normally associated with those of

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TABLE 1
Germination of Dehydrated Orchardgrass Somatic Embryos after Storage at 23°C for 0, 7, and 21 Days

Response[a]	Days of dehydrated storage[b]		
	0	7	21
No germination	126/28	333/74	396/88
Germination — no further growth	180/40	81/18	36/8
Germination — viable plants	144/32	36/8	18/4
Total	450/100	450/100	450/100

Note: Embryos were imbibed on solidified medium after test storage periods.

[a] Embryos that produced root hairs, roots, coleoptiles, and/or shoots, but failed to develop further were scored as germinated — no further growth. Those that produced green leaves and continued to grow were considered to be viable.
[b] Number of SEs/percentage of total SEs.

From Gray, D. J., Conger, B. V., and Songstad, D. D., In Vitro *Cell Dev. Biol.*, 23, 29, 1987. With permission.

seed embryos when proper environmental conditions are provided. High plant recovery rates suggest that dehydration followed by rehydration results in better regulation of embryo maturation and germination.

V. APPLICATIONS OF SYNTHETIC SEED FOR ORCHARDGRASS IMPROVEMENT

Difficulties associated with developing synthetic varieties have been so severe as to stifle the improvement of crops like orchardgrass.[52] The low value per plant and lack of suitable vegetative propagules combine to make seed the only choice for stand establishment. Although synthetic seed

FIGURE 5. Orchardgrass plant obtained from previously dehydrated SE. (From Gray, D. J., Conger, B. V., and Songstad, D. D., In Vitro *Cell Dev. Biol.*, 23, 29, 1987. With permission.)

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technology offers the lowest costs for vegetative propagation that can be envisioned, it is doubtful that synthetic seed costs can compete with natural seed costs for orchardgrass. A more efficient use for synthetic seed technology is in the development of improved orchardgrass varieties. It is currently not feasible to vegetatively propagate by division of tillers new genotypes destined to be utilized in foundation seed production. Synthetic seed would provide a cost-efficient method for cloning these lines. This would allow hybrid germplasm to be integrated into synthetic cultivars, resulting in a net increase of cultivar quality and a decrease in development time.

A remaining obstacle to implementing synthetic seed technology in this breeding scheme is that somatic embryogenesis in orchardgrass is genotype-dependent. However, the embryogenic trait was shown to be transmitted sexually through both male and female gametes, and expressed in approximately 50% of the F_1 plants of crosses between embryogenic and nonembryogenic parents.[53] The limited data suggest tetrasomic inheritance of a simple dominant trait. A genotype characterized by high-capacity somatic embryogenesis ("Embryogen-P") has been released and registered.[54] This genotype provides a source of germplasm that can be used to introgress the embryogenic trait into other breeding lines. Genetically diverse lines with high embryogenic capacity will further the application of synthetic seed technology for orchardgrass improvement.

ACKNOWLEDGMENTS

Partial support for this project was provided by the Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development under Grant No. DHR-5600-G-00-057-00. This is Florida Agricultural Experiment Station Journal Series No.R-02219

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American Journal of Botany 79(5): 542–546. 1992.

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM IMMATURE ZYGOTIC EMBRYOS OF MUSCADINE GRAPE (VITIS ROTUNDIFOLIA) CULTIVARS[1]

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Embryogenic cell lines of *Vitis rotundifolia* were produced from immature zygotic embryo explants obtained by culturing ovules, harvested at 20 d postanthesis, for 8 wk and then dissecting embryos from them. Ovules cultured on Nitsch and Nitsch medium with naphthoxyacetic acid and benzyladenine (BA) produced a brown exudate, necessitating three transfers to fresh medium at 2-wk intervals during the 8-wk culture cycle. Zygotic embryos that were subsequently isolated from cultured ovules and placed on the same medium produced a heterogenous callus from which eventually emerged embryogenic cell lines. A higher percentage of ovules from cultivars 'Dixie', 'Fry', 'Nesbitt', and 'Welder' produced zygotic embryos (31%–39%) than did those from 'Carlos' (3%). A higher percentage of 'Fry' ovules produced embryogenic lines from cultured zygotic embryos (6.3%) than did those of the other four cultivars (1%–1.6%). Embryogenic cell lines were white and composed of variably sized cell clusters, somatic embryos, and embryonic tissue embedded in a watery matrix. These lines were maintained for over 1 yr on modified Murashige and Skoog (MS) medium lacking growth regulators by transfer of selected cell clusters every 6 wk. White, opaque somatic embryos grew directly from cell clusters and passed through recognizable developmental stages. Germination was induced by transfer of somatic embryos to MS medium with BA. Although 80%–100% of embryos germinated, plant recovery was low due to poor shoot development.

Muscadine grape (*Vitis rotundifolia* Michx., subgenus *Muscadinia* Planch., $2n = 40$) is an important native fruit crop of the southeastern United States where it is used for fresh fruit, jelly, juice, and wine production (Gray and Benton, 1991). Improved cultivars have been produced via conventional breeding methods, but certain undesirable traits, such as thick skins and seededness, remain. Although thin skin and seedlessness are available in certain bunch grape cultivars (subgenus *Euvitis* Planch., $2n = 38$) and can be sexually transmitted within *Euvitis* (Gray et al., 1990), sexual compatibility between the subgenera is marginal due to differences in chromosome number (Jelenkovic and Olmo, 1969). This has made transfer of desirable traits from *Euvitis* to *Muscadinia* via sexual hybridization difficult (Goldy et al., 1988).

A possible alternative to the conventional approach is to utilize emerging biotechnologies such as somatic hybridization and/or recombinant DNA technology to accomplish transfer of these traits. However, application of either technique is dependent on availability of regenerative (i.e., embryogenic or organogenic) cell cultures. Both embryogenic and organogenic cell cultures have been developed for a number of bunch grape cultivars but have not been obtained for muscadine grapes (Gray and Meredith, in press). In this report, I describe long-lived embryogenic cell cultures from five muscadine grape cultivars. Although immature zygotic embryos (not desirable clonal tissue) are used here as explant tissue, development and manipulation of such embryogenic cell cultures from somatic tissues of the parent plant now seem within reach by extending and modifying the methodologies developed in this study. Such further advances may enable the use of biotechnical procedures for muscadine grape improvement.

MATERIALS AND METHODS

Initial efforts to isolate embryogenic cell lines from cultured zygotic embryos excised from mature seeds were unsuccessful. The extreme hardness of the mature grape seed coat made isolation of zygotic embryos difficult. This led to use of young zygotic embryos contained in soft, immature seeds (i.e., ovules obtained prior to wall hardening). The developmental stage at which soft ovules could be conveniently isolated was determined to be approximately 20 d after pollination. However, embryos that were large enough to be isolated with the aid of a stereomicroscope could not be found in ovules at this stage. Therefore, a two-stage system was employed to obtain and culture immature zygotic embryos.

The first stage employed in ovulo embryo rescue. Berries were harvested at approximately 20 d after anthesis. At this stage of development, visible embryos could not be found in a sample of 20 dissected immature ovules. Berries were surface disinfested for 5 min by agitation in 25% commercial bleach (1.3% NaOCl) containing a drop of Triton X surfactant, then rinsed twice in sterile distilled water. Ovules (2–5 mm in length) isolated from berries were dissected to eliminate one-fourth of the broad distal end (opposite from the micropyle), and two slices of tissue were taken to remove the rounded sides of each ovule as well. Ovules were then placed, micropyle end up and cut surfaces immersed, into N-1 medium (autoclaved Nitsch and Nitsch [1969] salts and vitamins with 3% sucrose, 0.2 mg/liter benzyladenine [BA], 1 mg/liter naphthoxyacetic acid [NOA], 0.7% TC agar [Hazelton Biologics, Lenexa, KS], pH 5.6 adjusted prior to adding agar and autoclaving). Sixteen ovules were placed into each 15 ×

[1] Received for publication 7 June 1991; revision accepted 21 January 1992.

The author thanks the Program in Science and Technology Cooperation, Office of the Science Advisor, United States Agency for International Development (grant #DHR-5600-G-00-0057-00) for providing partial support for this project. This is Florida Agricultural Experiment Station Journal Series No. R-01636.

100-mm sterile plastic petri dish containing 30 ml of medium, which was placed under a 16-hr cool-white fluorescent illumination (165 $\mu mol \cdot s^{-1} \cdot m^{-2}$) / 8-hr dark cycle at 25 C. After 2 wk, necrotic tissue was trimmed away and ovules were transferred to fresh medium. This step was repeated after 2 additional wk.

The second stage utilized zygotic embryos developed in stage 1 over a period of 5 wk. Ovules were dissected and examined for zygotic embryos which were removed and cultured, five per petri dish, directly on N-1 medium under the aforementioned environmental conditions. Embryos became visibly callused within 2 wk and were examined weekly for the emergence of embryogenic callus. Embryogenic callus was isolated and cultured on MMS medium (autoclaved Murashige and Skoog [1962] salts and vitamins minus glycine, with NH_4NO_3 and KNO_3 concentrations lowered to 412.5 g/liter and 475 g/liter, respectively, myo-inositol concentration raised to 1 g/liter and containing 6% sucrose, 2 g/liter washed [in distilled H_2O] activated charcoal, 0.7% TC agar, pH 5.6).

Genotypic differences among the cultivars 'Carlos', 'Dixie', 'Fry', 'Nesbitt', and 'Welder' were determined by culturing 64 ovules (96 for 'Welder') of each and then comparing recovery of zygotic embryos and embryogenic cell lines. This experiment consisted of a factorial arrangement of treatments in a completely random design. Replicates consisted of either single ovules or zygotic embryos. Data were analyzed with the Statistical Analysis System using a general linear model (SAS Institute, Inc., 1985).

Embryogenic cell lines were cultured on MMS medium, with transfer every 6 wk, to determine their relative longevity. Somatic embryos were germinated on MSB medium (autoclaved Murashige and Skoogs salts and vitamins, minus glycine, with 3% sucrose, 1 g/liter myo-inositol, 1 μM BA, 0.7% TC agar, pH 5.6). Plants (i.e., germinated somatic embryos with roots and shoots) were acclimated to greenhouse conditions as previously described for plants obtained via ovule culture (Gray et al., 1990).

RESULTS

Severe ovular browning occurred during in ovulo culture (stage 1). Brown material, presumably composed of tannins produced from wounding, diffused into the medium and formed a ring around each ovule, prompting excision of brown tissue and transfer of ovules to fresh medium after 2 wk. This step was repeated after 2 additional wk due to continued browning. Eventually, ovules became brown in color, although endosperm and zygotic embryos, if present, usually remained white.

Comparison of zygotic embryo recovery from ovules of five distinct cultivars showed that all exhibited an equivalent response, except for 'Carlos', which produced significantly fewer embryos (Table 1). Based on subjective evaluation, ovules of 'Carlos' produced more callus than the others; however, it was not possible to determine whether ovular callusing and zygotic embryo development were inversely related in this culture system.

Isolated zygotic embryos placed directly on N-1 medium produced a soft, friable heterogenous callus that was initially white to pale yellow in color but eventually turned brown to gray over the course of 4 wk. This callus was composed of cells of variable size and shape. Embryogenic callus was recovered from distinct sectors of the heterogenous callus over a period of 3 to 8 wk. White to yellow embryogenic calli were highly distinctive, usually emerging at a time when surrounding heterogenous callus was in a state of decline.

TABLE 1. *Effect of muscadine grape cultivar on recovery of zygotic embryos and embryogenic cell lines from cultured ovules*

Cultivar	Ovules	Zygotic embryos no. / %[a]	Embryogenic lines (%)
Carlos	64	2/3b[b]	1.6[c]/50a[d]
Dixie	64	20/31a	1.6/5c
Fry	64	25/39a	6.3/16b
Nesbitt	64	20/31a	1.6/5c
Welder	96	33/34a	1.0/3c

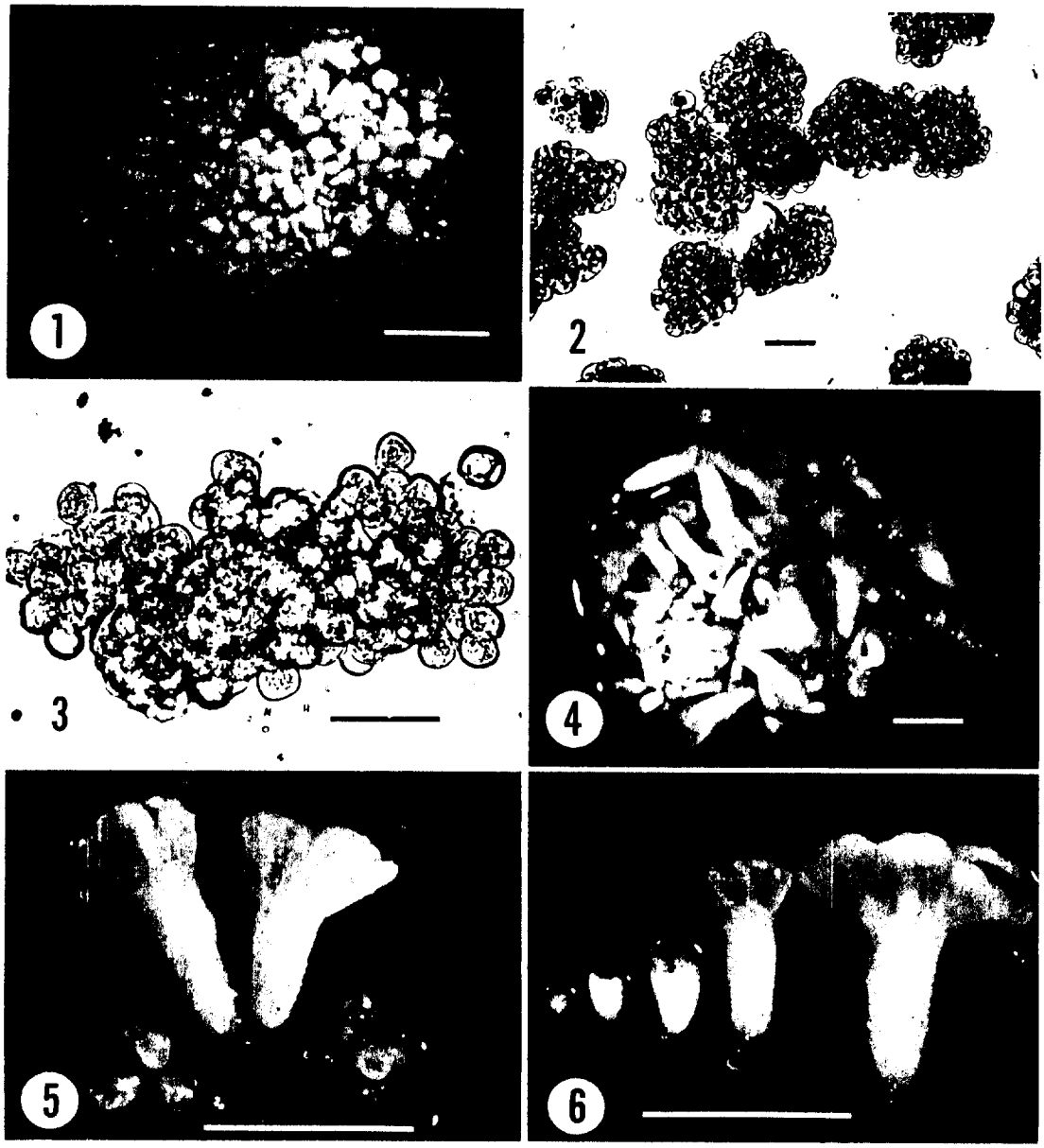
[a] Percentage based on number of zygotic embryos/number of ovules.
[b] Means with the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's New Multiple Range Test.
[c] Percentage based on number of embryogenic cell lines/number of ovules.
[d] Percentage based on number of embryogenic cell lines/number of zygotic embryos.

Comparison of embryogenic line recovery per ovule showed that 'Fry' exhibited a significantly higher response than the other cultivars tested (Table 1). When considering embryogenic line recovery per zygotic embryo explant, 'Carlos' was best with 'Fry' second. These latter results may be misleading, however, since a total of only two zygotic embryos were isolated from 'Carlos' ovules; one of these produced an embryogenic line.

Isolated embryogenic callus cultured on MMS medium was white to yellow in color and composed of cell clusters, somatic embryos, and amorphous masses of embryonic tissue embedded in a watery matrix material (Fig. 1). Cell clusters were highly variable in size and shape (Fig. 2). In contrast to the heterogenous callus produced on N-1 medium, the cell clusters that grew on MMS were composed of cells that were uniformly small, densely cytoplasmic, and isodiametric (Fig. 3). Embryogenic cultures were refined and maintained by careful selection of cell clusters and exclusion of embryos and amorphous masses during transfer. This could be easily accomplished by viewing cultures through a stereomicroscope and first removing undesirable material before transfer of the remainder of the culture to fresh medium. Under such transfer conditions, embryogenic cultures doubled in size every 2 wk and became increasingly uniform, being composed primarily of embryogenic cell clusters.

Somatic embryos developed continuously in these cultures; however, large masses of somatic embryos were produced by selective transfer of only the watery matrix material. This watery material contained very small cell clusters, from which subsequently developed masses of somatic embryos that were either single and unattached to other embryos (Fig. 4) or arranged in small fasciculate groups (Fig. 5). Embryos in fasciculate groups were attached to each other only at their extreme basal regions. Somatic embryos have continued to develop from regularly transferred cultures for over 1 yr.

Somatic embryos were always white and opaque and



Figs. 1–6. Somatic embryogenesis in *V. rotundifolia*. **1.** Embryogenic maintenance culture. Bar = 2 mm. **2.** Embryogenic cell clusters. Bar = 20 μm. **3.** Embryogenic cells. Bar = 20 μm. **4.** Somatic embryos produced from small cell clusters of cultures maintained for over 1 yr. Bar = 2 mm. **5.** Fasciculate development of somatic embryos. Bar = 2 mm. **6.** Somatic embryo developmental stages from left: proembryo, early torpedo, late torpedo, cotyledonary, enlarged cotyledonary. Note fusion of cotyledons on early torpedo and cotyledonary stages. Bar = 2 mm.

most had recognizable hypocotyls and cotyledons. However, misshapen amorphous masses of embryonic tissue, not morphologically recognizable as somatic embryos except for color and texture, were also commonly recovered (e.g., Fig. 1). Somatic embryos were faithful in morphology to grape zygotic embryos, except for frequent occurrence of fused cotyledons (Figs. 5, 6). Fusion of cotyledons was often so severe as to form a complete ring around the distal hypocotyl tip. Other than this abnormality, all stages typical of embryo development were routinely recovered from embryogenic cultures (Fig. 6).

Germination of somatic embryos (germination is defined here as the sequence of events leading to enlargement and greening of the embryo axis) did not occur readily on MMS medium. However, transfer of single embryos to MSB resulted in rapid germination. On MSB, 80%–100% of cotyledonary embryos germinated within 7 d (data not shown). Germinated embryos underwent rapid enlargement in conjunction with a progressive change in pigmentation from white-to-yellow-to-green. Cotyledons became leafy and root emergence typically occurred (Fig. 7). However, very few germinated embryos produced shoots despite prolonged culture on MSB as well as tests using media containing various growth regulators. To date only four plants from somatic embryos of the cultivar 'Welder' have been recovered. These plants grow vigorously and exhibit a normal vegetative morphology (Fig. 8). Due to the long grape life cycle, reproductive mor-

Fig. 7. Germinated somatic embryo of *V. rotundifolia*. Bar = 2 mm.

phology cannot be evaluated until after 2–3 additional yr of growth.

DISCUSSION

This report for *V. rotundifolia* extends the range of grape species for which somatic embryogenesis has been reported into subgenus *Muscadinia*. Within subgenus *Euvitis*, somatic embryogenesis has been reported for three species, *Vitis longii* Prince, *Vitis rupestris* Scheele, and *Vitis vinifera* L., as well as a number of their hybrids (Gray and Meredith, in press). Zygotic embryo explants were previously used to initiate cultures of *V. longii* and *V. vinifera*; however, in ovulo culture was not required. This culture step was found to be a convenient method of obtaining large numbers of zygotic embryos. Use of N-1 medium to induce somatic embryogenesis was previously reported for *Euvitis* (Gray and Meredith, in press); this medium is similar to that used in many other reports concerning induction of somatic embryogenesis in that a phenoxy-herbicide combined with a cytokinin are utilized as growth regulators. Development of embryogenic cultures from all five cultivars tested is encouraging when viewed in light of previous reports for *Euvitis* where distinct genotypic differences have been noted that limit somatic embryogenesis to a few cultivars (Rajasekaran and Mullins, 1983; Stamp and Meredith, 1988a, b; Gray and Meredith, in press).

Maintenance of embryogenic cell cultures on medium devoid of growth regulators has not been documented for grape. Previously, long-term maintenance of grape embryogenesis was accomplished either through direct secondary embryogenesis on growth regulator-free medium

Fig. 8. Plant derived from *V. rotundifolia* somatic embryo.

(Krul, 1985; Gray and Mortensen, 1987) or through cell culture on medium containing a phenoxy-herbicide (Gray and Mortensen, 1987; Stamp and Meredith, 1988a; Matsuta and Hirabayashi, 1989). Maintenance of embryogenic cells on growth regulator-free medium is a logical combination of the two methodologies but required stringent selection of proper culture material in order to achieve stable regenerative cultures. These growth regulator-independent cultures differ from typical embryogenic grape callus (e.g., Gray and Mortensen, 1987) by being homogeneously composed of embryogenic cells. The embryogenic grape cell cultures resemble those previously reported for *Daucus carota* L. (Smith and Krikorian, 1989, 1990) in cell morphology and autotrophic growth characteristics but do not require short (2-wk) subculture intervals to maintain rapid growth.

Increase of culture mass in this system occurs by division and growth of embryogenic cells contained in clusters. Fragmentation of the growing clusters results in continual formation of new embryogenic material. Somatic embryos grow from the embryogenic cell clusters and, if

not physically disturbed, remain connected via a narrowed suspensorlike structure. This is shown in Fig. 5, where two embryos are connected to each other through the initial cell cluster. Thus, the embryogenic cell clusters represent proembryonal complexes as defined by Haccius (1978). This interpretation differs from that of Smith and Krikorian (1990), who considered similar cell masses of *D. carota* to be preglobular stage embryos that develop directly into somatic embryos. In grape, the embryogenic cell clusters do not exhibit intercellular organization typical of embryogenesis. Further, developing somatic embryos that grow from the cell clusters are common and recognizable from an early stage of development (e.g., Fig. 6).

Somatic embryo morphology in *V. rotundifolia* was similar to that of *Euvitis* species, except for the frequent occurrence of fused cotyledons. Although pluricotyly and similar fusion occur in *Euvitis*, embryos with normal cotyledonary morphology are also common (Gray and Mortensen, 1987). Cotyledonary fusion clearly begins at a very early stage of embryo development, since a complete ring of young cotyledonary tissue often can be seen on early torpedo stage embryos (see Fig. 5, second embryo from left). Ammirato (1987) described similar cotyledonary fusion as a common abnormality of somatic embryos, which frequently is associated with a decreased development of the shoot. This would explain our observation of poor shoot development during germination. Changes in culture protocol to modify early cotyledon development, such that only two discrete regions of cotyledonary tissue form on the distal embryo apex, would improve plant recovery rates.

Use of medium containing BA was previously documented for germination of grape somatic embryos (Gray, 1987, 1989). Both somatic and zygotic embryos of grape are dormant; they remain white and do not germinate without a dormancy-breaking pretreatment (Gray, 1989). With zygotic embryos contained in seeds, cold stratification is an effective pretreatment, whereas BA or dehydration are effective for somatic embryos. Although 80%–100% germination was obtained in the present study, very few plants were recovered. However, low plant recovery rate is due to poor shoot development which, in turn, can be attributed to faulty development of shoot apical initials, as explained above, and not due to a failure of germination.

This study has demonstrated that embryogenic cultures derived from zygotic embryos of *V. rotundifolia* (subgenus *Muscadinia*) resemble and perform similarly to those initiated from both zygotic and vegetative tissue of *Euvitis* species. This suggests that *V. rotundifolia* will be amenable to various biotechnical procedures once induction of embryogenesis from vegetative tissues is achieved.

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Effect of Ovule Maturity on Recovery of Zygotic Embryos and Embryogenic Cultures from Muscadine Grape

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Additional index words. Vitis rotundifolia, embryo rescue, ovule culture, somatic embryogenesis

Embryogenic and organogenic cell cultures have been reported for several bunch grape cultivars (Gray and Meredith, 1992). Recently, embryogenesis was obtained for the first time in muscadine grape (*Vitis rotundifolia* Michx., subgenus *Muscadinia* Planch.) (2n = 40) using a unique ovule culture protocol to obtain zygotic embryos, which were then used as explants (Gray, 1992). In this report, we describe the effects of ovule maturity and genotype on the recovery of zygotic embryos and embryogenic cultures from 11 muscadine grape cultivars.

Cultivar vines at the Central Florida Research and Education Center's experimental vineyard in Leesburg were evaluated for floral development daily during Apr. and May 1991. Flower clusters in which anthesis had occurred in ≈25% of individual flowers were tagged to establish anthesis date. Ovule maturities were based on age in days postanthesis (DPA) (10, 20, 30, and 40). No fewer than 130 ovules for each treatment (each cultivar at each sample date) were dissected from berries and cultured as described by Gray (1992); however, random losses due to contamination resulted in an actual sample range of 109 to 127 ovules among treatments. After 5 weeks in culture, ovules were dissected and the presence of zygotic embryos was determined. To produce embryogenic cultures, ovules from 10-day samples were recultured intact, since cursory examination revealed that zygotic embryos large enough to be recognized through a stereo microscope were not present after 5 weeks. With 10-DPA samples, we found that zygotic embryos, embryogenic tissue, or both, when present, emerged from ovules that ruptured during culture. Zygotic embryos from 20, 30, and 40 DPA were cultured to induce somatic embryogenesis as described by Gray (1992). Differences in percentage of ovules with zygotic embryos and percentage of ovules forming embryogenic cultures due to cultivar differences or ovule maturity were determined statistically using a factorial arrangement of treatments in a completely random design.

When data from all sample dates were combined, cultivars differed in the percentage of ovules that contained zygotic embryos (Table 1). A significantly higher percentage of 'Dixie' ovules produced zygotic embryos than those of the other cultivars, except 'Summit'. 'Summit' was intermediate in response but not significantly different from 'Dixie' or 'Regale'. A similar statistically significant overlapping range of responses was evident among all cultivars. Relative ovule maturity also affected zygotic embryo recovery when responses from all cultivars were combined (Table 1). Ovules sampled at 40 DPA produced significantly more zygotic embryos than younger ovules; ovules sampled at 30 DPA responded better than those at 10 or 20 DPA. In previous research, 'Carlos' produced fewer zygotic embryos than 'Dixie', 'Fry', 'Nesbitt', or 'Welder' when only the 20-DPA ovule sample date was tested (Gray, 1992). There was a significant interaction between cultivar and ovule maturity for zygotic embryo recovery (Table 1) due to one instance when ovules from 'Welder' were more responsive at 10 DPA (13% ± 3%) than 20 DPA (4% ± 2%) and equal in response at 30 DPA (9% ± 3%).

There was no difference in the percentage of embryogenic culture lines obtained among cultivars (Table 1). Previously, it was determined that 20-DPA 'Fry' ovules produced significantly more embryogenic cultures than those of 'Carlos', 'Dixie', 'Nesbitt', or 'Welder' (Gray, 1992). However, when compared to the previous tests, ≈10× more replicates per treatment were used in the present study, a procedure that may have led to better statistical resolution. Ovules sampled at 20, 30, or 40 DPA were similar but produced significantly more embryogenic cultures than those sampled at 10 DPA. This result demonstrates that ovule or zygote maturity is not critical in obtaining embryogenic cultures.

Table 1. Effects of cultivar and sample date on recovery of zygotic embryos and embryogenic cultures from muscadine grape ovules.

Factors	Ovules tested (no.)	Zygotic embryos (%)	Embryogenic cultures (%)
Cultivar			
Dixie	461	21 a[z]	0.9 a
Summit	467	18 ab	1.1 a
Regale	474	15 bc	0.4 a
Carlos	502	13 c	1.6 a
Welder	487	13 c	1.4 a
Fry	472	10 cd	1.3 a
Triumph	459	9 de	1.3 a
Magnolia	496	9 de	0.6 a
Nesbitt	464	7 ef	0.7 a
Noble	486	7 ef	0.4 a
Alachua	506	6 f	0.8 a
DPA[y]			
10	1397	1.4 c	0.07 b
20	1324	2.7 c	1.1 ab
30	1271	8.0 b	1.0 ab
40	1300	35.0 a	1.8 a
Main effects			
Cultivar (CV)		*	NS
Sample time (ST)		*	*
CV × ST		*	NS

[z]Mean separation in columns by analysis of standard errors from categorical modeling (SAS, 1988).
[y]DPA = days postanthesis.
[NS,*]Nonsignificant or significant at $P = 0.05$, respectively.

Newly initiated embryogenic cultures, which resembled those of *Vitis longii* Prince (Gray and Mortensen, 1987), were placed on growth regulator-free medium and grown as described by Gray (1992). Somatic embryos were germinated on Murashige and Skoog basal medium (Gray, 1992), and resulting plants were acclimated to greenhouse conditions. Representative plants of all cultivars except 'Nesbitt' were established in greenhouse pots. To date, all plants appear normal; they exhibit typical *V. rotundifolia* vegetative morphology, but several years are required to evaluate reproductive morphology. Immature ovules are convenient explants when compared to seeds, which have hard seedcoats that make removing zygotic embryos difficult.

Received for publication 13 Aug. 1992. Accepted for publication 19 Oct. 1992. This project was supported in part by the Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development, grant DHR-5600-G-00-0057-00. Florida Agricultural Experiment Station no. R-02616. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product and does not imply its approval to the exclusion of other products or vendors that also may be suitable. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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Reprints from HortScience, Vol. 28(3), March 1993
A Publication of the American Society for Horticultural Science, Alexandria, VA 22314

Reprinted from

Proc. Fla. State Hort. Soc. 106:124-128. 1993.

SUCROSE, ABSCISIC ACID AND METHYLGLYOXAL BIS-(GUANYLHYDRAZONE) AFFECT GRAPE SOMATIC EMBRYOGENESIS

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Additional index words. Vitis, synthetic seeds, controlling embryogenesis, tissue culture.

Abstract. **The effects of sucrose, abscisic acid (ABA) and methylglyoxal bis-(guanylhydrazone) (MGBG) on grape (*Vitis vinifera* L. cv. Thompson Seedless) somatic embryogenesis was examined by subculturing somatic embryos and embryogenic cells to somatic embryo maintenance medium (EMM) containing either 60, 90, 120, 150, or 180 g/liter sucrose; 0, 1, 10 or 100 M μABA; or 0, 0.1, 1 or 10 mM MGBG. The number of cotyledonary stage somatic embryos resembling zygotic embryos was increased by culturing embryogenic cells on EMM with 120 g/liter sucrose for 2 to 3 months. No difference was detected between the control (60 g/liter) and the other sucrose treatments. ABA (10-100 μM) reduced precocious germination of cotyledonary and torpedo stage somatic embryos without reducing embryo viability. The same ABA concentrations reduced secondary embryo production among cotyledonary stage embryos but not torpedo stage embryos. ABA inhibited plant formation from cotyledonary stage somatic embryos, but promoted plantlet regeneration from torpedo stage embryos at the 1 μM concentration. Adding 1 or 10 mM MGBG to EMM inhibited the growth of grape embryogenic cells and somatic embryos. The number of cotyledonary stage somatic embryos resembling zygotic embryos was reduced 88% to 100% when embryogenic cultures were incubated on EMM with 1 or 10 mM MGBG, respectively, for 3 months.**

Somatic embryos have historically been used for clonal propagation of elite lines (Attree et al., 1990), or to obtain genetically engineered plants following infection with *Agrobacterium tumefaciens* (Chee, 1990; Delbreil et al., 1993) or bombardment with DNA-coated tungsten particles (Cao et al., 1992; Vasil et al., 1992). Somatic embryos have also been used as "synthetic seeds", either encapsulated in alginate (Redenbaugh et al., 1987) or fluid-drilling gel (Kitto et al., 1991), dehydrated naked (Gray, 1989; Gray et al., 1987), or dehydrated following encapsulation (Kitto and Janick, 1985a,b; Kim and Janick, 1989; Janick et al., 1989).

[1]Florida Agricultural Experiment Station Journal Series No. N-008037. The authors thank Karen Kelly for her technical assistance. Partial support for this project was provided by the Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development under Grant No. DHR-5600-G-00-0057-00.

Plants have been obtained from dehydrated somatic embryos of alfalfa [*Medicago sativa* L. (Anandarajah and McKersie, 1990b; Senaratna et al., 1989)], grape [*Vitis* sp. (Gray, 1989)], maize [*Zea mays* L. (Compton et al., 1992)] and orchardgrass [*Dactylis glomerata* L. (Gray et al., 1987)]. In addition to clonal propagation and genetic transformation of important hybrid cultivars, synthetic seeds of grape could be used for germplasm conservation. Currently, grape germplasm is maintained in vineyards, exposing accessions to insects and diseases, and possible destruction by natural disasters. A synthetic seed system would allow us to store grape accessions in seed repositories as clonal somatic embryogenic cultures or dehydrated, quiescent somatic embryos (Gray and Compton, 1993). This would eliminate the need for extensive germplasm collections in vineyards.

In order to have an efficient synthetic seed system, an embryo to plant regeneration system must be in place. Plant regeneration from grape somatic embryos is often difficult (Gray, 1989). Dehydration of grape somatic embryos improved plant formation (Gray, 1989), but the percentage of embryos that formed plants was low (~30%). Plant regeneration of alfalfa (Anandarajah and McKersie, 1990a,b; Senaratna et al., 1989) and maize (Green, 1982; Green et al., 1983; Petersen et al., 1992) was improved by culturing somatic embryos on medium with high osmoticum and ABA. Increased plant regeneration resulted from an improvement in somatic embryo quality. In grape, the production of high quality somatic embryos is relatively low. Subculturing grape somatic embryos to medium with high osmoticum and/or ABA may improve embryo quality.

Polyamines have been implicated as a possible cause of abnormal somatic embryo development in grape (Faure et al., 1991). The addition of polyamine biosynthesis inhibitors, such as MGBG, to EMM may restore normal embryo development and plantlet formation. MGBG is a polyamine biosynthesis inhibitor that blocks spermidine and spermine synthesis by inhibiting the decarboxylation of S-adenosylmethionine (Galston and Kaur-Sawhney, 1987). Therefore, it may be possible to improve grape somatic embryo quality by adding MGBG to the culture medium.

The objective of this work was to examine the effects of sucrose, ABA and MGBG on grape somatic embryo growth and development, and to identify treatments that improve somatic embryo quality and plantlet development.

Materials and Methods

Establishment of embryogenic cultures. Embryogenic cultures of 'Thompson Seedless' grape were established using a method adapted from Gray (1989) and Gray and Mortensen (1987). Briefly, 1 to 5 mm leaf explants were excised from shoot cultures maintained on modified C_2D medium (Chee et al., 1984) with 5 μM benzyladenine (BA; Sigma Chemical Co., St. Louis, MO) and incubated in 100 x 15 mm plastic petri plates containing 25 ml of somatic embryo induction medium (Gray, 1989; Gray and Mortensen, 1987) for 6 weeks in the dark at 25C. Embryogenic callus and somatic embryos were transferred to 100 x 15 mm petri plates containing 50 ml of somatic embryo maintenance medium [modified Murashige and Skoog medium (1962) with (per liter) 412.5 mg NH_4NO_3 and 475 mg KNO_3 (instead of 1650 and 1900 mg, respectively), 1 g myo-inositol, 60 g sucrose and 7 g TC agar (JRH Biosciences, Lenexa, KS) at pH 5.4; EMM] and a 16 h photoperiod (0.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from cool-white fluorescent lamps). Embryogenic callus and cells were subcultured to fresh EMM monthly.

Unless otherwise stated, embryogenic cultures representing globular, heart, and torpedo stage somatic embryos plus embryogenic cells were transferred to 100 x 15 mm petri plates that contained 50 ml of test medium. There were five plates per treatment, each with five clumps of embryogenic material weighing approximately 15 mg. Cultures were at least 6 months old when used. Culture fresh weight and the number of cotyledonary embryos that resembled zygotic embryos were recorded each month. Culture dry weight was measured at the end of the experiment. Cultures were dried in an oven at 70C for 72 h.

Effects of sucrose on the growth and development of embryogenic cells. The effects of sucrose on grape somatic embryogenesis was studied by subculturing embryogenic cells and somatic embryos (heart and globular stage) to EMM containing 60, 90, 120, 150, or 180 g/liter sucrose. Embryogenic cultures were subcultured monthly for 3 months.

Effects of ABA on somatic embryo germination, secondary embryo production, and plant formation. The effect of ABA on somatic embryogenesis was evaluated by subculturing somatic embryos, representing all developmental stages, to EMM containing 120 g/liter sucrose and either 0, 1, 10, or 100 μM ABA. ABA was filter-sterilized and added to cooled, autoclaved medium. Cotyledonary stage embryos (16 per plate) were laid individually on the medium surface whereas torpedo stage embryos were cultured in groups of 10 with five groups per plate. Heart and globular stage embryos were cultured in clumps as mentioned above. There were four plates per treatment. Data on precocious germination (as determined by hypocotyl and root elongation), embryo viability (as determined by the ability to stain pink after tetrazolium treatment), and the number of secondary somatic embryos were recorded for cotyledonary and torpedo stage embryos after 4 weeks. The number of plants obtained from each treatment was recorded 4 weeks after transfer to germination medium [MS with (per liter) 30 g sucrose, 1 g myo-inositol, 7 g TC agar and 1 μM BA; pH 5.7 (Gray and Mortensen, 1987)]. Data recorded for heart and globular stage embryos included the number of cotyledonary, torpedo, heart, and globular stage embryos per plate after 4 weeks.

Effects of MGBG on somatic embryogenesis. The effects of MGBG on grape somatic embryogenesis was examined by subculturing embryogenic cells to EMM containing 120 g/liter sucrose and MGBG at 0, 0.1, 1, or 10 mM concentrations. MGBG was filter-sterilized and added to cooled, autoclaved medium.

Experimental design and statistical analysis. Treatments for all experiments were arranged in a completely randomized design with subsampling. Statistical analysis was conducted using the GLM procedure of the statistical analysis system (SAS, 1988). Percentage data were transformed using arcsin prior to analysis and converted back to the original scale using $p = (\sin p')^2$ (Bartlett, 1937; Zar, 1984). Comparisons of each treatment with the experimental control were made using Dunnett's procedure (1955).

Results and Discussion

Effects of sucrose on the growth and development of embryogenic cells. During the first month of culture, embryogenic cells and somatic embryos grown on standard EMM (60 g/liter sucrose) had greater fresh weights than those on EMM supplemented with 90 to 180 g/liter sucrose (Table 1). After the first month, no difference in fresh weight was observed among embryos grown on EMM with 60 g/liter sucrose and those reared on EMM with 90 or 120 g/liter sucrose. Embryos grown on EMM with 150 to 180 g/liter sucrose weighed less than the controls. Embryogenic cultures maintained on EMM with 90 or 120 g/liter sucrose had greater dry weights than embryos grown on the standard EMM medium, whereas embryos maintained on EMM with 150 to 180 g/liter sucrose had reduced dry weights.

Raising the sucrose concentration of EMM failed to increase the number of cotyledonary stage embryos that resembled their zygotic counterparts during the first month of culture (Table 1). However, the quality of cotyledonary stage somatic embryos was improved by maintaining embryogenic cultures on EMM with 120 g/liter sucrose for 2 to 3 months. Embryo quality was not improved by growing embryogenic cultures on EMM with 90, 150, or 180 g/liter sucrose.

The moisture content of grape somatic embryos grown on EMM with 60, 90, 120, 150, and 180 g/liter sucrose was 88%, 77%, 77%, 73%, and 72%, respectively. This suggests that grape somatic embryos grown on EMM with high sucrose contained less unbound water than similar embryos grown on standard EMM. Improvement in quality of embryos reared on EMM with 120 g/liter sucrose may be related to partial dehydration during embryo maturation. Others have found that transferring somatic embryos to medium with high sucrose improved embryo quality and subsequent plant regeneration (Anandarajah and McKersie, 1990a,b; Petersen et al., 1992; Senaratna et al., 1989).

Effects of ABA on somatic embryo germination, secondary embryo production, and plant formation. ABA dramatically affected the growth and development of grape embryogenic cell cultures. Adding 10 or 100 μM ABA to EMM inhibited precocious germination and the number of secondary embryos produced on cotyledonary stage embryos when compared to similar embryos incubated on standard EMM (Table 2). No differences were observed between cotyledonary stage embryos incubated on EMM with 0 or 1 μM ABA. Precocious germination of torpedo stage somatic embryos was promoted by adding 1 μM ABA to EMM but was inhibited by higher (10 and 100 μM) concentrations. The percentage of torpedo stage somatic embryos that produced secondary embryos and the number of secondary embryos per somatic embryo were not affected by ABA. Plantlet formation from cotyledonary embryos was inhibited by ABA. In contrast, the ability of torpedo stage embryos to develop into plants was doubled by incubating embryos on EMM with 1 μM ABA when compared to embryos incubated on EMM lacking ABA. Higher ABA concentrations (10 and 100 μM) inhibited plantlet formation from torpedo stage embryos. Incubating heart or globular stage embryos on EMM with ABA failed to promote embryo maturation and plantlet development (data not shown).

ABA inhibited the growth of heart and globular stage somatic embryos as indicated by the lower number of embryos produced per plate when compared to similar embryos on EMM lacking ABA (Table 3). Somatic embryo maturation was also affected by the ABA concentration in the medium. At 10 μM ABA, fewer heart or globular stage somatic embryos matured to the cotyledonary stage, whereas the percentages of torpedo, and heart and globular stage embryos were similar to the controls. At 100 μM ABA, the percentage of cotyledonary stage embryos that developed from heart stage embryos was reduced, the percentage of torpedo stage embryos was increased, and the percentage of heart and globular stage embryos was less than the controls. The maturation of globular stage embryos to the cotyledonary stage was inhibited by 10 and 100 μM ABA. However, the percentages of torpedo, and heart and globular stage embryos were similar to the controls.

ABA improved plantlet formation from somatic embryos of maize (Green, 1982; Green et al., 1983; Petersen et al., 1992), alfalfa (Anandarajah and McKersie, 1990b; Senaratna et al., 1989) and spruce (Attree et al., 1990). We observed that ABA (1 μM) promoted plantlet development from torpedo stage somatic embryos of grape, but was ineffective in controlling precocious germination and secondary embryo production. In addition, ABA inhibited plantlet formation from cotyledonary embryos and maturation of heart and globular stage embryos. This suggests that torpedo stage embryos are the best material for ABA treatment and plant development. However, further studies are required to identify an ABA concentration that promotes plantlet development but inhibits precocious germination and secondary embryo production.

Effects of MGBG on somatic embryogenesis. The addition of 10 mM MGBG to EMM inhibited the growth of embryogenic cultures during the first month of culture (Table 4). Adding 1 or 10 mM MGBG resulted in a significant reduction in culture fresh weight and dry weight including

Table 1. Effect of sucrose on the growth and development of somatic embryogenic cell cultures of 'Thompson Seedless' grape.

Sucrose (g/liter)	Culture fresh wt (mg)						Culture dry wt (mg)		No. cotyledonary embryos					
	1 mo.		2 mo.		3 mo.		3 mo.		1 mo.		2 mo.		3 mo.	
60	2811		1873	ns	1947		230		0.4		0.8		0	
90	2141	*	2042	ns	1783	ns	408	*	4.0	ns	3.0	ns	5.8	ns
120	1809	*	1673	ns	1615	ns	366	*	5.0	ns	7.4	*	13.2	*
150	809	*	755	*	699	*	186	ns	0	ns	4.6	ns	3.4	ns
180	613	*	565	*	684	*	187	ns	0	ns	0	ns	0	ns

*Significantly different from the control (60) according to Dunnett's at the 0.05 level; ns = nonsignificant.

Table 2. Effects of ABA on somatic embryo germination, secondary embryo production and plant formation from cotyledonary and torpedo stage somatic embryos of 'Thompson Seedless' grape.

Embryo stage[z]	ABA conc (μM)	Precocious germination (%)		Second embryo production (%)[y]		Second embryo production Number[x]		Plant formation[w] (%)	
C	0	97.0	ns	50		57.8		13	(4/32)
	1	88.0	ns	56	ns	59.0	ns	3	(1/32)
	10	1.5	*	26	ns	7.0	*	0	(0/32)
	100	1.5	*	39	ns	13.8	*	0	(0/32)
T	0	65.0		84		47.3		6	(5/80)
	1	81.0	*	77	ns	23.2	ns	11	(9/80)
	10	0.5	*	65	ns	7.5	ns	0	(0/80)
	100	0.5	*	74	ns	10.0	ns	1	(1/80)

[z]There were 64 (cotyledonary) and 80 (torpedo) embryos per treatment.
[y]The percent of embryos that formed secondary somatic embryos.
[x]Number of secondary embryos per somatic embryo.
[w] Embryos were transferred to modified MS medium with 1 μM BA for germination and plant development.
*Significantly different from the control (0 ABA) according to Dunnett's at the 0.05 level; ns = nonsignificant.

Table 3. Effect of ABA on the development of heart and globular stage somatic embryos of 'Thompson Seedless' grape.

Embryo stage	ABA conc (μM)	Number of embryos[z]		Embryo developmental stages: Cotyledonary %[y]		Torpedo %		Heart/ Globular %	
H	0	317		8.6		38.6		52.9	
	1	228	*	10.2	ns	48.8	ns	41.0	ns
	10	161	*	0.1	*	55.0	ns	45.0	ns
	100	149	*	0.1	*	69.8	*	30.2	*
G	0	568		4.5		44.7		50.8	
	1	352	*	5.3	ns	48.1	ns	46.7	ns
	10	182	*	0.5	*	36.7	ns	62.8	ns
	100	153	*	0.1	*	52.8	ns	47.2	ns

[z]Average number of somatic embryos per plate after 4 weeks.
[y]Percent of the total number of embryos per plate.
*Significantly different from the control (0 ABA) according to Dunnett's at the 0.05 level; ns = nonsignificant.

Table 4. Effect of MGBG on the growth and development of somatic embryogenic cultures of 'Thompson Seedless' grape.

MGBG conc (mM)	Culture fresh wt (mg) 1 mo.		Culture fresh wt (mg) 2 mo.		Culture fresh wt (mg) 3 mo.		Culture dry wt (mg) 3 mo.		No. cotyledonary embryos 1 mo.		No. cotyledonary embryos 2 mo.		No. cotyledonary embryos 3 mo.	
0	1809		1673	ns	1676		374		3.0		2.6		3.3	
0.1	1769	ns	1844	ns	1946	ns	439	*	4.0	ns	4.4	ns	3.4	ns
1	1528	ns	1429	*	471	*	190	*	5.2	ns	0.8	ns	0.4	*
10	850	ns	319	*	0	*	0		0	ns	0	ns	0	*

[z]Number of cotyledonary stage empbryos resembling zygotic embryos.
*Significantly different from the control (0 MGBG) according to Dunnett's at the 0.05 level; ns = nonsignificant.

the death of cells incubated on EMM with 10 mM MGBG by the third month. The production of normal grape somatic embryos was not affected by MGBG during the first 2 months of culture. However, the formation of cotyledonary stage embryos was inhibited after 3 months by 1 to 10 mM MGBG. No difference in the number of high quality cotyledonary embryos was detected among embryogenic cultures incubated on EMM with 0 or 0.1 mM MGBG throughout the course of the experiment.

Previous studies have suggested that the production of abnormal grape somatic embryos may be related to an increase in endogenous polyamine synthesis during the torpedo stage of development (Faure et al., 1991). MGBG inhibits the synthesis of two polyamines, spermidine and spermine, by blocking the decarboxylation of S-adenosylmethionine (Galston and Kaur-Sawhney, 1987). Polyamine and ethylene synthesis are interrelated, i.e., ethylene production elevates when polyamine synthesis is blocked and decreases when it is not (Galston and Kaur-Sawhney, 1987). MGBG has been shown to increase ethylene synthesis while inhibiting polyamine production (Roberts et al., 1984). Therefore, we do not know at present if the growth inhibition of grape embryogenic cells was caused by the addition of MGBG or by a possible increase in endogenous ethylene production.

This study demonstrates that grape somatic embryogenesis can be controlled using sucrose, ABA, and a specific polyamine biosynthesis inhibitor. The production of high quality somatic embryos was promoted by doubling the sucrose concentration of the standard medium currently used for grape somatic embryogenesis. Adding low concentrations (1 μM) of ABA improved plantlet formation from torpedo stage embryos but was ineffective in controlling precocious germination and secondary embryo formation. Further studies are required in order to identify an ABA concentration that controls precocious germination and secondary embryo formation without inhibiting plantlet formation. MGBG inhibited the growth and development of grape somatic embryos at concentrations above 0.1 mM but failed to improve the quality of grape somatic embryos. This suggests that polyamines may play a role in regulating the growth of grape somatic embryogenic cultures. Further investigation in the area of endogenous polyamine production may reveal inhibitors that promote the production of high quality grape somatic embryos without inhibiting embryo growth and development.

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Reprinted from:
Proc. Fla. State Hort. Soc. 107: 308-310. 1994.

MICROPROPAGATION OF 'SOUTHERN HOME' HYBRID GRAPE

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Additional index words. *Vitis* sp., axillary bud proliferation, tissue culture.

Abstract. **Stems collected from plants grown in the vineyard were trimmed to 2 cm and all fully-expanded leaves removed before surface-disinfestation in 25% chlorine bleach for 5 or 10 min and 6 rinses with an antibiotic solution. Shoot apices (1-3 mm) with 1 or 2 leaf primordia were cultured for 4 weeks on Murashige and Skoog medium with either 5 μM benzyladenine (BA), or 1.7 μM indole-3-acetic acid (IAA), 0.3 μM gibberellic acid (GA_3) and 0.5 μM kinetin. Fewer explants were contaminated following 10 min surface disinfestation (5%) compared to 5 min (30%). More explants produced shoots when cultured on medium containing BA (88%) than on medium with IAA, GA_3, and kinetin (57%). Shoot tips cultured in medium with BA produced an average of 3 axillary shoots every 5 weeks. From 76% to 83% of the axillary shoots rooted when transferred to medium with 1 μM naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA), respectively. Fewer (43%) shoots produced roots when transferred to medium without auxin. Approximately 90% of 'Southern Home' plantlets survived acclimatization to ambient conditions regardless of the rooting treatment. This study indicates that shoot-tip micropropagation would be an excellent way to rapidly increase the number of 'Southern Home' plants available for wholesale and retail buyers.**

'Southern Home' is a complex interspecific hybrid grape (*Vitis rotundifolia* Michx., *V. munsoniana* Simpson ex Munson, *V. popenoei*, *V. vinifera* L.) with deeply-cut, attractive leaves, and self-fertile flowers that fruit in moderately loose clusters (Mortensen et al., 1994). 'Southern Home' appears to be immune to Pierce's disease (Mortensen et al., 1977), and nearly immune to anthracnose [*Elsinoe ampelina* (de Bary) Shear] and downy mildew [*Plasmospara viticola* (B & C) Berl. & de T.]. It is highly resistant to ripe rot [*Glomerella cingulata* (Stonem) Spaulding & von Schrenk], bitter rot [*Melanconium fuligineum* (Scrib. & Viala) Cav.], and black rot [*Guignardia bidwellii* (Ell.) Viala & Ravaz]. Color instability makes 'Southern Home' unsuitable for wine production (Mortensen et al., 1994). However, its attractive foliage and clusters of large berries with firm flesh make this grape an excellent variety for the home garden.

To facilitate the rapid release of 'Southern Home' to wholesale and retail markets a micropropagation scheme that rapidly produced a large number of propagules would be beneficial. Previous work suggests that micropropagation can be used to rapidly propagate a wide variety of bunch and muscadine species and cultivars (Chee et al., 1984; Gray and Benton, 1991; Gray and Fisher, 1985; Gray and Klein, 1987, 1989; Lee and Wetzstein, 1990). The purpose of this study was to evaluate the potential of micropropagation as a means of rapidly propagating 'Southern Home' plants for wholesale and retail growers.

Materials and Methods

Explant preparation and culture establishment. Explants were prepared as previously described by Gray and Benton (1991). Briefly, 2 cm shoot tips were excised from 'Southern Home' vines grown in the experimental vineyard at the Central Florida Research and Education Center in Leesburg, placed between layers of moist paper towel, and transferred to the laboratory where all fully expanded leaves and tendrils were removed. Shoot tips were surface-disinfested in a bleach solution (1.25% NaClO plus 1 drop per 100 ml Triton X-100 surfactant) for 5 or 10 min before 6 rinses with an antibiotic

Florida Agricultural Experiment Station Journal Series No. N-00993. Partial support for this project was provided by the Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development under Grant No. DHR-5600-G-00-0057-00.

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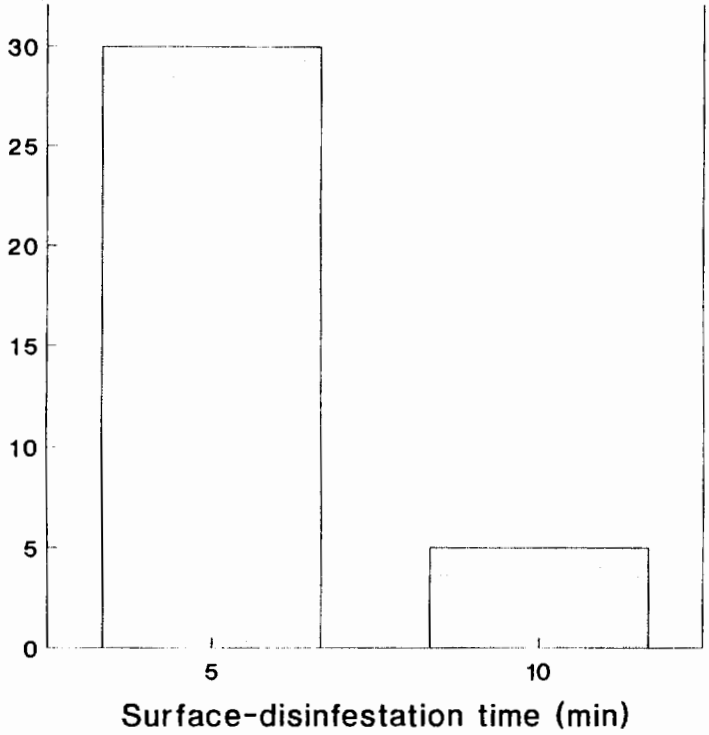

Figure 1. Percentage of contaminated explants following surface-disinfestation for 5 or 10 min in a mild bleach solution. Means were significantly different according to Maximum Likelihood Analysis of Variance.

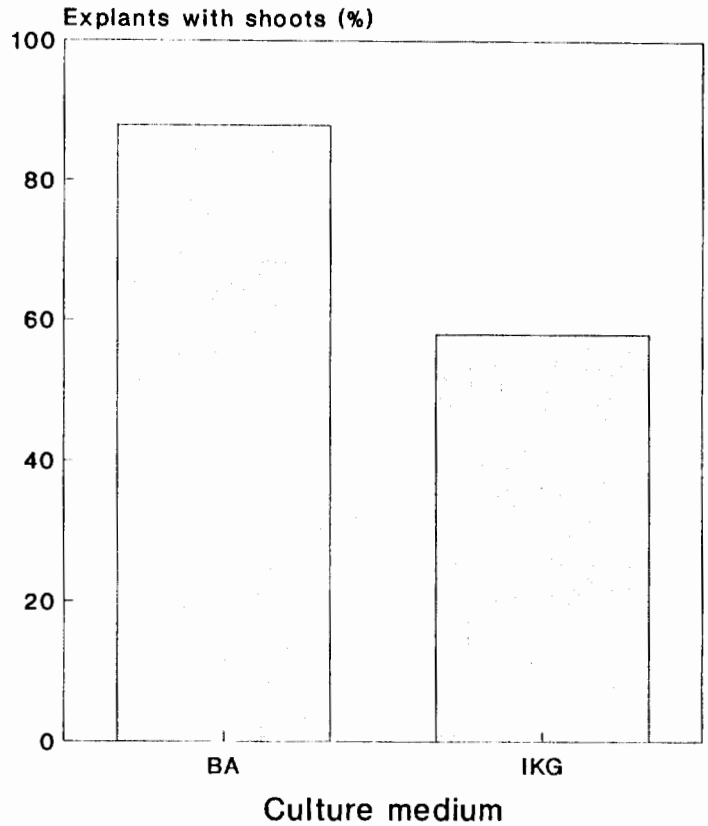

Figure 2. Effect of culture medium on the percentage of explants that produced shoots. Explants were cultured on shoot proliferation medium with either 5 µM BA or 1.7µM IAA, 0.5 µM kinetin and 0.3 µM GA_3 (IKG) for 4 weeks. Means were significantly different according to Maximum Likelihood Analysis of Variance.

solution (20 mg/l Rifampicin, Sigma Chemical Co., St. Louis, MO). Shoot tips were stored in petri plates on moistened, sterile paper towels. Explants consisted of the meristem plus 1 or 2 leaf primordia (1-3 mm total length) and were cultured in 100 × 15 mm petri plates that contained 25 ml of modified MS medium [Murashige and Skoog salts (1962) with (per liter) 204 g KH_2PO_4 (instead of 170 g), 0.5 mg nicotinic acid, 0.5 mg pyridoxine HCl, 0.1 mg thiamine HCl, 30 g sucrose, 1 g myo-inositol, and 7 g TC agar (JRH Biosciences, Lenexa, KS) at pH 5.5] supplemented with 5 µM BA or 1.7 µM IAA, 0.3 µM GA_3 and 0.5 µM kinetin for 4 weeks. There were 2 explants per plate and 10 plates per treatment.

Shoot tips longer than 1 cm were transferred to Magenta GA_7 vessels (Magenta Corp., Chicago, IL) that contained 50 ml of shoot proliferation medium [C_2D (Chee et al., 1984) with (per liter) 204 g KH_2PO_4 (instead of 170 g), 1 mg nicotinic acid, 1 mg pyridoxine HCl, 1 mg thiamine HCl, 30 g sucrose, 1 g myo-inositol, 3 µM BA (Sigma Chemical Co., St. Louis, MO), 20 mg Rifampicin, and 8 g TC agar (JRH Biosciences, Lenexa, KS) at pH 5.5] for 6 weeks. There were 5 explants per GA_7 vessel and a total of 10 vessels.

Elongated shoots (2-3 cm) were transferred to rooting medium (MS as above) with 1 µM of either IBA or NAA, or no auxin (control) for 3 weeks. Rooted shoots were transferred to cell packs (4.0 × 5.5 cm with 72 cells per flat) filled with (by volume) 1 part Pro Mix BX (Premier Brands, Inc., New Rochelle, NY) to 1 part coarse vermiculite, covered with a clear plastic lid, and grown under the same conditions as tissue cultures until new shoot growth appeared (1 week). Plants were acclimatized to ambient conditions by gradually removing the lid over 5 days. Plants were misted manually 3 to 4 times daily with distilled water to avoid desiccation. Acclimatized plants were moved to the greenhouse 1 week later and incubated under natural light and photoperiod.

Explants were arranged in a completely randomized design with subsampling. Data included the percentage of contaminated explants (4 weeks), percentage of explants that produced shoots (4 weeks), number of shoots per explant (10 weeks), percentage of explants with roots and number of roots per explant (3 weeks), and the percentage of explants that survived acclimatization (2 weeks). Numerical data were analyzed by analysis of variance and means compared using Ryan-Einot-Gabriel-Welsch Multiple Range Test. Percentage data were analyzed using Maximum Likelihood Analysis of Variance and means compared using the standard error of the mean.

Results and Discussion

The length of surface-disinfestation significantly influenced the percentage of contaminated shoot-tip explants (Fig. 1). Fewer explants became contaminated following 10 min surface-disinfestation compared with the 5 min treatment. Previous work indicated that a 3 min surface-disinfestation time was best for establishing shoot-tip cultures of various bunch and muscadine grape cultivars (Gray and Benton, 1991; Gray and Klein, 1989). This year (1994) was damp in central Florida and the increased moisture and humidity may have promoted fungal and bacteria spore populations making it necessary to use longer surface-disinfestation times. This indicates that the optimal length of surface-disinfestation of shoot tips from field grown plants may vary depending on the cultivar and disease pressure during a particular year.

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Figure 3. 'Southern Home' shoot-tip culture 9 weeks after initiation on medium with 5 µM BA.

The growth regulators present in the medium significantly influenced the percentage of explants that produced shoots (Fig. 2). More explants produced shoots when cultured in medium containing BA compared with those cultured in medium containing IAA, kinetin, and GA_3. This supports previous work with bunch and muscadine grape which demonstrated that shoot-tip explants of these species respond best when cultured in medium with BA (Chee et al., 1984; Gray and Benton, 1991; Gray and Klein, 1989). Shoot tips (1 cm) initiated in medium with BA were transferred to fresh medium after 4 weeks in vitro. These explants proliferated rapidly and produced an average of 3.3 shoots per explant every 5 weeks during the 10 week test (Fig. 3). This value falls within the range of shoot proliferation rates reported for bunch and muscadine grapes (Gray and Benton, 1991; Gray and Klein, 1989).

Shoots from 'Southern Home' cultures rooted in medium containing auxin. More shoots developed roots when transferred to medium with 1 µM of either IBA or NAA compared with those transferred to medium lacking exogenous auxin

Table 1. Percentage of 'Southern Home' microshoots that rooted, number of roots per shoot, and percentage of plants acclimatized to ambient conditions.

Auxin[z]	N[y]	Shoots with roots (%)	No. roots per shoot
IBA	30	83.3 ± 6.8	4.5 a
NAA	30	76.7 ± 7.7	3.1 a
NONE	30	43.3 ± 9.0	0.8 b

[z]Shoots were rooted in MS medium with 1 µM of either IBA or NAA, or no auxin (NONE).
[y]Number of shoots transferred to rooting medium.
± Values represent the standard error of the mean.
Means within columns with the same letter are not different according to Ryan-Einot-Gabriel-Welsch Multiple Range Test.

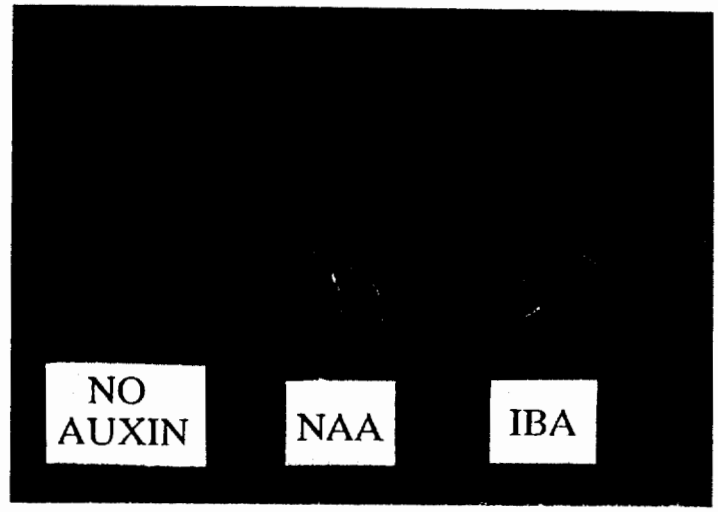

Figure 4. 'Southern Home' plantlets rooted on medium without auxin, 1 µM NAA, or 1 µM IBA for 3 weeks.

(Table 1). In addition, shoots rooted in medium with auxin produced more roots than those rooted in medium without auxin (Fig. 4). This is similar to previous reports on rooting of bunch and muscadine grape microshoots (Gray and Benton, 1991; Gray and Klein, 1989; Lee and Wetzstein, 1990). Gray and Benton (1991) demonstrated that tissue culture-derived grape shoots could be rooted directly in potting mix without an in vitro rooting step. Although not tested here, direct rooting of 'Southern Home' shoots in soil would alleviate a major culture step and may increase micropropagation efficiency.

Approximately 90% of 'Southern Home' plantlets were acclimatized to ambient conditions in the laboratory and survived transfer to the greenhouse. There was no effect of rooting treatment on the ability of 'Southern Home' plantlets to survive acclimatization (data not shown).

Based on current shoot proliferation, rooting, and acclimatization data, over 2000 acclimatized 'Southern Home' plants could be produced every 16 weeks from an initial stock of 25 shoot-tip explants. Therefore, shoot-tip micropropagation could be used to efficiently increase the number of 'Southern Home' plants, and because grafting is not required, micropropagated plants could be planted directly to the field.

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Reprinted from:
Proc. Fla. State Hort. Soc. 107: 311-312. 1994.

A COMPARISON OF SHOOT MICROPROPAGATION AMONG BUNCH AND MUSCADINE GRAPE SPECIES AND CULTIVARS

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Additional index words. *Vitis*, plant propagation, tissue culture.

Abstract. **Micropropagation of *Vitis bourquiniana* Lenoir 'Black Spanish', *Vitis champini* Planchon 'Dog Ridge', *Vitis* hybrids ('Blanc du Bois', 'Himrod', and 'Niagara Seedless'), *Vitis rotundifolia* Michx. ('Carlos' and 'Dixie'), and *Vitis vinifera* L. ('Autumn Seedless', 'Cabernet Sauvignon', 'Carignane', 'French Colombard', 'Ruby Cabernet', and 'Tokay') was accomplished. Shoot tips taken from micropropagated plants in long-term culture were axenically inoculated onto solidified C_2D medium containing 5 µM benzyladenine. Culture times consisting of either one or two 4-week cycles were compared for effect on shoot number. Shoot production was genotype-specific, such that a range in response among cultivars tested was noted. The best responding variety was *V. champini* 'Dog Ridge' with 5.8 shoots per apex and the worst was *V. rotundifolia* 'Dixie' with 2.2 shoots per apex. All other varieties exhibited an intermediate response. When shoot micropropagation from nodal explants and apices was compared, however, no significant difference was noted. In vitro micropropagation offers rapid clonal production of desirable grape varieties and is a source of sterile leaf explant material for initiating embryogenic cultures, which, in turn, are useful targets for genetic transformation.**

In vitro shoot micropropagation is used for rapid clonal production of desirable genotypes and is well established for bunch and muscadine grape varieties (Chee et al.,1984; Gray and Fisher,1985; Gray and Benton,1990). In addition, micropropagated shoot cultures provide a constant and reliable source of sterile leaf explant material for generating embryogenic cultures. Embryogenic cultures are useful in grape transformation studies (Gray and Meredith,1992).

Our study explored three facets of grape shoot proliferation. Using well-established long-term cultures, we compared shoot proliferation rates among 13 grape varieties initiated from apices, monitored the effect of culture time, and compared the number of shoots originating from nodal explants with the number derived from apical meristems.

Materials and Methods

Plant material. Shoot apical meristem explants were obtained from greenhouse-grown grape plants. Plants were maintained under a fungicidal spray program that alternated benomyl, mancozeb, and 1-(4-Chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone (Bayleton, Mobay Corp), at the manufacturer's recommended labelled rate. Excised shoot tips (ca. 5 mm in length) from greenhouse plants were wrapped in moistened towels and collected in polyethylene bags. Shoot tips were surface sterilized in a 25% commercial bleach solution (1.3% NaOCl) containing 1 drop of Triton surfactant for 3 min with agitation, followed by 3 sterile rinses in distilled water.

Shoot micropropagation. Meristems (0.5-1 mm diameter) were axenically removed and placed, cut surface down, on modified C_2D medium (Chee and Pool,1982) containing 5 µM of 6-benzyladenine (BA), 7 g/l^{-1} agar (JRH Biosciences, Product #90-500-550), and 30 g/l^{-1} sucrose. Medium pH was adjusted to 5.8 with potassium hydroxide prior to sterilization in an autoclave for 20 min at 121C. Apices and nodal explants, approximately 5 mm in length, were micro-dissected from 13 micropropagated grape cultivars. All micropropagated cultures were initiated during Apr. 1992 and July 1993, and were maintained on a sporadic transfer schedule until Feb. 1994, at which time they were transferred to fresh medium in anticipation of this experiment.

Apices and nodal material (containing axillary buds) were inoculated onto 100 × 25 mm petri dishes, maintaining fidelity of origin. Each of 5 plates contained 5 explants of either apical meristems or nodes. Cultures were maintained under cool-white fluorescent lights, 18 hr (light):6 hr (dark) and 28C. The number of shoots produced by each explant was determined after each 4-week cycle.

Experiments. In the first experiment differences in shoot production among 13 cultivars, and between two consecutive 4-week cycles were compared. Fifty apices were established under the conditions described. The species and cultivars tested were *Vitis bourquiniana* 'Black Spanish', *Vitis champini* 'Dog Ridge', *Vitis rotundifolia* 'Carlos' and 'Dixie', *Vitis vinifera* 'Autumn Seedless', 'Cabernet Sauvignon', 'Carignane', 'French Colombard', 'Ruby Cabernet', and 'Tokay', and three complex U.S. hybrids, 'Blanc du Bois', 'Himrod', and 'Niagara Seedless'. Cultures were grown for 4 weeks, and the number of shoots per apex determined. The most vigorous shoot tips were chosen to reinitiate the next 4-week cycle, after which the number of shoots per apex was determined again.

In the second experiment, we compared the effect of explant source on shoot production. Nodal explants and apical meristems were excised from cultures of *V. rotundifolia* 'Dixie' and 'Carlos' and 4 bunch-type varieties: 'Blanc du Bois', 'Carignane', 'French Colombard', and 'Niagara Seedless'. Five explants from each species were inoculated onto C_2D medium, which contained 5 µM of BA. A total of 25 replicates of apical meristem buds and lateral buds were initiated from each of the 6 grape species. Cultures were maintained under lights, as described above, for 4 weeks. After 4 weeks, proliferating shoots were counted.

Data were subjected to the Student-Newman-Keuls means analysis procedure of the Statistical Analysis System (SAS, 1988).

Results and Discussion

Shoot data were collected at 2 consecutive 4-week culture cycles (Table 1). Of 13 cultivars evaluated from apices, *V.*

Florida Agricultural Experiment Station Journal Series No. N-01037. Partial support for this project was provided by the Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development under Grant No. DHR-5600-G-00-0057-00.

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Table 1. Average in vitro shoot production from *Vitis* cultivars.

Vitis species and cultivars	No. apices[z]	Shoots/apex[y]
V. bourquiniana		
'Black Spanish'	50	3.2cdef
V. champini		
'Dog Ridge'	50	5.8a
V. rotundifolia		
'Carlos'	50	4.3bc
'Dixie'	30	2.2f
V. vinifera		
'Autumn Seedless'	50	2.7def
'Cabernet Sauvignon'	50	4.3bc
'Carignane'	50	2.5ef
'French Colombard'	50	4.6b
'Ruby Cabernet'	40	3.7bcde
'Tokay'	50	3.4bcdef
Eastern U.S. *Vitis* Hybrids		
'Blanc du Bois'	50	4.0bcd
'Himrod'	50	4.5b
'Niagara Seedless'	49	3.5bcdef

[z]Data combined from 2 consecutive 4-week culture cycles on C_2D medium with 5μM BA.
[y]Means within columns with the same letter are not significantly different at $\alpha = 0.01$ according to Student-Newman-Keuls test.

champini 'Dog Ridge', a useful nematode-resistant rootstock variety, produced longer shoots and the most shoots per apex (5.8). *V. rotundifolia* 'Dixie', a muscadine variety that developed long stems and large leaves, had the fewest shoots per apex (2.2), but was statistically indistinguishable from six other varieties. However, in an earlier in vitro shoot proliferation study, 'Dixie' produced more multiple shoots (4.0 shoots per apex) over 2 and 3 culture periods (Gray and Fisher,1985), suggesting that additional culture cycles might be responsible for the increased shoot proliferation rate. Lee and Wetzstein (1990) reported that the rate of muscadine shoot proliferation was slow during culture establishment for the first 8 weeks, regardless of BA levels, but later shoots increased rapidly.

Both axillary bud and apical meristem explant sources were compared for 6 *Vitis* cultivars. Laterals and apices did not differ in their ability to produce shoots (data not shown). However, in micropropagation, apices are preferable material from which to initiate disease-free grape cultures. For example, apical meristems are useful in the exclusion of endemic *Agrobacterium tumefaciens* in bunch grape cultivars (Burr et al.,1988) and Pierce's disease in muscadine stock plants (Robacker and Chang,1992).

When we compared shoot proliferation rates between 2 consecutive 4-week culture cycles, we found that significantly more shoots were produced in the second cycle. In the first cycle, 3.4 shoots per apex ($\underline{n} = 334$) were produced, whereas, in the second cycle, 4.0 shoots per apex ($\underline{n} = 335$) were produced. One possible explanation for increased shoot proliferation lies in the culture transfer history. Since initiation in Jan. 1992, most of the cultures had been transferred to fresh C_2D medium at 1-month intervals. At the time of this experiment, however, a few cultures were rescued for the first time in 5 months. Therefore, the increase in shoot production between the first and second culture intervals may have been due to these cultures overcoming the growth lag associated with inadequate transfers. Selection of uniformly aged and consistently transferred cultures might ensure consistent shoot proliferation rates in grape micropropagation.

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Proc. Fla. State Hort. Soc. 106:124-128. 1993.

SUCROSE, ABSCISIC ACID AND METHYLGLYOXAL BIS-(GUANYLHYDRAZONE) AFFECT GRAPE SOMATIC EMBRYOGENESIS

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Abstract. **The effects of sucrose, abscisic acid (ABA) and methylglyoxal bis-(guanylhydrazone) (MGBG) on grape (*Vitis vinifera* L. cv. Thompson Seedless) somatic embryogenesis was examined by subculturing somatic embryos and embryogenic cells to somatic embryo maintenance medium (EMM) containing either 60, 90, 120, 150, or 180 g/liter sucrose; 0, 1, 10 or 100 M μABA; or 0, 0.1, 1 or 10 mM MGBG. The number of cotyledonary stage somatic embryos resembling zygotic embryos was increased by culturing embryogenic cells on EMM with 120 g/liter sucrose for 2 to 3 months. No difference was detected between the control (60 g/liter) and the other sucrose treatments. ABA (10-100 μM) reduced precocious germination of cotyledonary and torpedo stage somatic embryos without reducing embryo viability. The same ABA concentrations reduced secondary embryo production among cotyledonary stage embryos but not torpedo stage embryos. ABA inhibited plant formation from cotyledonary stage somatic embryos, but promoted plantlet regeneration from torpedo stage embryos at the 1 μM concentration. Adding 1 or 10 mM MGBG to EMM inhibited the growth of grape embryogenic cells and somatic embryos. The number of cotyledonary stage somatic embryos resembling zygotic embryos was reduced 88% to 100% when embryogenic cultures were incubated on EMM with 1 or 10 mM MGBG, respectively, for 3 months.**

Somatic embryos have historically been used for clonal propagation of elite lines (Attree et al., 1990), or to obtain genetically engineered plants following infection with *Agrobacterium tumefaciens* (Chee, 1990; Delbreil et al., 1993) or bombardment with DNA-coated tungsten particles (Cao et al., 1992; Vasil et al., 1992). Somatic embryos have also been used as "synthetic seeds", either encapsulated in alginate (Redenbaugh et al., 1987) or fluid-drilling gel (Kitto et al., 1991), dehydrated naked (Gray, 1989; Gray et al., 1987), or dehydrated following encapsulation (Kitto and Janick, 1985a,b; Kim and Janick, 1989; Janick et al., 1989).

[1]Florida Agricultural Experiment Station Journal Series No. N-008037. The authors thank Karen Kelly for her technical assistance. Partial support for this project was provided by the Program in Science and Technology Cooperation, Office of the Science Advisor, U.S. Agency for International Development under Grant No. DHR-5600-G-00-0057-00.

Plants have been obtained from dehydrated somatic embryos of alfalfa [*Medicago sativa* L. (Anandarajah and McKersie, 1990b; Senaratna et al., 1989)], grape [*Vitis* sp. (Gray, 1989)], maize [*Zea mays* L. (Compton et al., 1992)] and orchardgrass [*Dactylis glomerata* L. (Gray et al., 1987)]. In addition to clonal propagation and genetic transformation of important hybrid cultivars, synthetic seeds of grape could be used for germplasm conservation. Currently, grape germplasm is maintained in vineyards, exposing accessions to insects and diseases, and possible destruction by natural disasters. A synthetic seed system would allow us to store grape accessions in seed repositories as clonal somatic embryogenic cultures or dehydrated, quiescent somatic embryos (Gray and Compton, 1993). This would eliminate the need for extensive germplasm collections in vineyards.

In order to have an efficient synthetic seed system, an embryo to plant regeneration system must be in place. Plant regeneration from grape somatic embryos is often difficult (Gray, 1989). Dehydration of grape somatic embryos improved plant formation (Gray, 1989), but the percentage of embryos that formed plants was low (~30%). Plant regeneration of alfalfa (Anandarajah and McKersie, 1990a,b; Senaratna et al., 1989) and maize (Green, 1982; Green et al., 1983; Petersen et al., 1992) was improved by culturing somatic embryos on medium with high osmoticum and ABA. Increased plant regeneration resulted from an improvement in somatic embryo quality. In grape, the production of high quality somatic embryos is relatively low. Subculturing grape somatic embryos to medium with high osmoticum and/or ABA may improve embryo quality.

Polyamines have been implicated as a possible cause of abnormal somatic embryo development in grape (Faure et al., 1991). The addition of polyamine biosynthesis inhibitors, such as MGBG, to EMM may restore normal embryo development and plantlet formation. MGBG is a polyamine biosynthesis inhibitor that blocks spermidine and spermine synthesis by inhibiting the decarboxylation of S-adenosylmethionine (Galston and Kaur-Sawhney, 1987). Therefore, it may be possible to improve grape somatic embryo quality by adding MGBG to the culture medium.

The objective of this work was to examine the effects of sucrose, ABA and MGBG on grape somatic embryo growth and development, and to identify treatments that improve somatic embryo quality and plantlet development.

Materials and Methods

Establishment of embryogenic cultures. Embryogenic cultures of 'Thompson Seedless' grape were established using a method adapted from Gray (1989) and Gray and Mortensen (1987). Briefly, 1 to 5 mm leaf explants were excised from shoot cultures maintained on modified C_2D medium (Chee et al., 1984) with 5 μM benzyladenine (BA; Sigma Chemical Co., St. Louis, MO) and incubated in 100 x 15 mm plastic petri plates containing 25 ml of somatic embryo induction medium (Gray, 1989; Gray and Mortensen, 1987) for 6 weeks in the dark at 25C. Embryogenic callus and somatic embryos were transferred to 100 x 15 mm petri plates containing 50 ml of somatic embryo maintenance medium [modified Murashige and Skoog medium (1962) with (per liter) 412.5 mg NH_4NO_3 and 475 mg KNO_3 (instead of 1650 and 1900 mg, respectively), 1 g myo-inositol, 60 g sucrose and 7 g TC agar (JRH Biosciences, Lenexa, KS) at pH 5.4; EMM] and a 16 h photoperiod (0.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from cool-white fluorescent lamps). Embryogenic callus and cells were subcultured to fresh EMM monthly.

Unless otherwise stated, embryogenic cultures representing globular, heart, and torpedo stage somatic embryos plus embryogenic cells were transferred to 100 x 15 mm petri plates that contained 50 ml of test medium. There were five plates per treatment, each with five clumps of embryogenic material weighing approximately 15 mg. Cultures were at least 6 months old when used. Culture fresh weight and the number of cotyledonary embryos that resembled zygotic embryos were recorded each month. Culture dry weight was measured at the end of the experiment. Cultures were dried in an oven at 70C for 72 h.

Effects of sucrose on the growth and development of embryogenic cells. The effects of sucrose on grape somatic embryogenesis was studied by subculturing embryogenic cells and somatic embryos (heart and globular stage) to EMM containing 60, 90, 120, 150, or 180 g/liter sucrose. Embryogenic cultures were subcultured monthly for 3 months.

Effects of ABA on somatic embryo germination, secondary embryo production, and plant formation. The effect of ABA on somatic embryogenesis was evaluated by subculturing somatic embryos, representing all developmental stages, to EMM containing 120 g/liter sucrose and either 0, 1, 10, or 100 μM ABA. ABA was filter-sterilized and added to cooled, autoclaved medium. Cotyledonary stage embryos (16 per plate) were laid individually on the medium surface whereas torpedo stage embryos were cultured in groups of 10 with five groups per plate. Heart and globular stage embryos were cultured in clumps as mentioned above. There were four plates per treatment. Data on precocious germination (as determined by hypocotyl and root elongation), embryo viability (as determined by the ability to stain pink after tetrazolium treatment), and the number of secondary somatic embryos were recorded for cotyledonary and torpedo stage embryos after 4 weeks. The number of plants obtained from each treatment was recorded 4 weeks after transfer to germination medium [MS with (per liter) 30 g sucrose, 1 g myo-inositol, 7 g TC agar and 1 μM BA; pH 5.7 (Gray and Mortensen, 1987)]. Data recorded for heart and globular stage embryos included the number of cotyledonary, torpedo, heart, and globular stage embryos per plate after 4 weeks.

Effects of MGBG on somatic embryogenesis. The effects of MGBG on grape somatic embryogenesis was examined by subculturing embryogenic cells to EMM containing 120 g/liter sucrose and MGBG at 0, 0.1, 1, or 10 mM concentrations. MGBG was filter-sterilized and added to cooled, autoclaved medium.

Experimental design and statistical analysis. Treatments for all experiments were arranged in a completely randomized design with subsampling. Statistical analysis was conducted using the GLM procedure of the statistical analysis system (SAS, 1988). Percentage data were transformed using arcsin prior to analysis and converted back to the original scale using $p = (\sin p')^2$ (Bartlett, 1937; Zar, 1984). Comparisons of each treatment with the experimental control were made using Dunnett's procedure (1955).

Results and Discussion

Effects of sucrose on the growth and development of embryogenic cells. During the first month of culture, embryogenic cells and somatic embryos grown on standard EMM (60 g/liter sucrose) had greater fresh weights than those on EMM supplemented with 90 to 180 g/liter sucrose (Table 1). After the first month, no difference in fresh weight was observed among embryos grown on EMM with 60 g/liter sucrose and those reared on EMM with 90 or 120 g/liter sucrose. Embryos grown on EMM with 150 to 180 g/liter sucrose weighed less than the controls. Embryogenic cultures maintained on EMM with 90 or 120 g/liter sucrose had greater dry weights than embryos grown on the standard EMM medium, whereas embryos maintained on EMM with 150 to 180 g/liter sucrose had reduced dry weights.

Raising the sucrose concentration of EMM failed to increase the number of cotyledonary stage embryos that resembled their zygotic counterparts during the first month of culture (Table 1). However, the quality of cotyledonary stage somatic embryos was improved by maintaining embryogenic cultures on EMM with 120 g/liter sucrose for 2 to 3 months. Embryo quality was not improved by growing embryogenic cultures on EMM with 90, 150, or 180 g/liter sucrose.

The moisture content of grape somatic embryos grown on EMM with 60, 90, 120, 150, and 180 g/liter sucrose was 88%, 77%, 77%, 73%, and 72%, respectively. This suggests that grape somatic embryos grown on EMM with high sucrose contained less unbound water than similar embryos grown on standard EMM. Improvement in quality of embryos reared on EMM with 120 g/liter sucrose may be related to partial dehydration during embryo maturation. Others have found that transferring somatic embryos to medium with high sucrose improved embryo quality and subsequent plant regeneration (Anandarajah and McKersie, 1990a,b; Petersen et al., 1992; Senaratna et al., 1989).

Effects of ABA on somatic embryo germination, secondary embryo production, and plant formation. ABA dramatically affected the growth and development of grape embryogenic cell cultures. Adding 10 or 100 μM ABA to EMM inhibited precocious germination and the number of secondary embryos produced on cotyledonary stage embryos when compared to similar embryos incubated on standard EMM (Table 2). No differences were observed between cotyledonary stage embryos incubated on EMM with 0 or 1 μM ABA. Precocious germination of torpedo stage somatic embryos was promoted by adding 1 μM ABA to EMM but was inhibited by higher (10 and 100 μM) concentrations. The percentage of torpedo stage somatic embryos that produced secondary embryos and the number of secondary embryos per somatic embryo were not affected by ABA. Plantlet formation from cotyledonary embryos was inhibited by ABA. In contrast, the ability of torpedo stage embryos to develop into plants was doubled by incubating embryos on EMM with 1 μM ABA when compared to embryos incubated on EMM lacking ABA. Higher ABA concentrations (10 and 100 μM) inhibited plantlet formation from torpedo stage embryos. Incubating heart or globular stage embryos on EMM with ABA failed to promote embryo maturation and plantlet development (data not shown).

ABA inhibited the growth of heart and globular stage somatic embryos as indicated by the lower number of embryos produced per plate when compared to similar embryos on EMM lacking ABA (Table 3). Somatic embryo maturation was also affected by the ABA concentration in the medium. At 10 μM ABA, fewer heart or globular stage somatic embryos matured to the cotyledonary stage, whereas the percentages of torpedo, and heart and globular stage embryos were similar to the controls. At 100 μM ABA, the percentage of cotyledonary stage embryos that developed from heart stage embryos was reduced, the percentage of torpedo stage embryos was increased, and the percentage of heart and globular stage embryos was less than the controls. The maturation of globular stage embryos to the cotyledonary stage was inhibited by 10 and 100 μM ABA. However, the percentages of torpedo, and heart and globular stage embryos were similar to the controls.

ABA improved plantlet formation from somatic embryos of maize (Green, 1982; Green et al., 1983; Petersen et al., 1992), alfalfa (Anandarajah and McKersie, 1990b; Senaratna et al., 1989) and spruce (Attree et al., 1990). We observed that ABA (1 μM) promoted plantlet development from torpedo stage somatic embryos of grape, but was ineffective in controlling precocious germination and secondary embryo production. In addition, ABA inhibited plantlet formation from cotyledonary embryos and maturation of heart and globular stage embryos. This suggests that torpedo stage embryos are the best material for ABA treatment and plant development. However, further studies are required to identify an ABA concentration that promotes plantlet development but inhibits precocious germination and secondary embryo production.

Effects of MGBG on somatic embryogenesis. The addition of 10 mM MGBG to EMM inhibited the growth of embryogenic cultures during the first month of culture (Table 4). Adding 1 or 10 mM MGBG resulted in a significant reduction in culture fresh weight and dry weight including

Table 1. Effect of sucrose on the growth and development of somatic embryogenic cell cultures of 'Thompson Seedless' grape.

Sucrose (g/liter)	Culture fresh wt (mg)			Culture dry wt (mg)	No. cotyledonary embryos		
	1 mo.	2 mo.	3 mo.	3 mo.	1 mo.	2 mo.	3 mo.
60	2811	1873 ns	1947	230	0.4	0.8	0
90	2141 *	2042 ns	1783 ns	408 *	4.0 ns	3.0 ns	5.8 ns
120	1809 *	1673 ns	1615 ns	366 *	5.0 ns	7.4 *	13.2 *
150	809 *	755 *	699 *	186 ns	0 ns	4.6 ns	3.4 ns
180	613 *	565 *	684 *	187 ns	0 ns	0 ns	0 ns

*Significantly different from the control (60) according to Dunnett's at the 0.05 level; ns = nonsignificant.

Table 2. Effects of ABA on somatic embryo germination, secondary embryo production and plant formation from cotyledonary and torpedo stage somatic embryos of 'Thompson Seedless' grape.

Embryo stage[z]	ABA conc (μM)	Precocious germination (%)	Second embryo production (%)[y]	Second embryo production Number[x]	Plant formation[w] (%)
C	0	97.0 ns	50	57.8	13 (4/32)
	1	88.0 ns	56 ns	59.0 ns	3 (1/32)
	10	1.5 *	26 ns	7.0 *	0 (0/32)
	100	1.5 *	39 ns	13.8 *	0 (0/32)
T	0	65.0	84	47.3	6 (5/80)
	1	81.0 *	77 ns	23.2 ns	11 (9/80)
	10	0.5 *	65 ns	7.5 ns	0 (0/80)
	100	0.5 *	74 ns	10.0 ns	1 (1/80)

[z]There were 64 (cotyledonary) and 80 (torpedo) embryos per treatment.
[y]The percent of embryos that formed secondary somatic embryos.
[x]Number of secondary embryos per somatic embryo.
[w] Embryos were transferred to modified MS medium with 1 μM BA for germination and plant development.
*Significantly different from the control (0 ABA) according to Dunnett's at the 0.05 level; ns = nonsignificant.

Table 3. Effect of ABA on the development of heart and globular stage somatic embryos of 'Thompson Seedless' grape.

Embryo stage	ABA conc (μM)	Number of embryos[z]	Embryo developmental stages: Cotyledonary %[y]	Torpedo %	Heart/Globular %
H	0	317	8.6	38.6	52.9
	1	228 *	10.2 ns	48.8 ns	41.0 ns
	10	161 *	0.1 *	55.0 ns	45.0 ns
	100	149 *	0.1 *	69.8 *	30.2 *
G	0	568	4.5	44.7	50.8
	1	352 *	5.3 ns	48.1 ns	46.7 ns
	10	182 *	0.5 *	36.7 ns	62.8 ns
	100	153 *	0.1 *	52.8 ns	47.2 ns

[z]Average number of somatic embryos per plate after 4 weeks.
[y]Percent of the total number of embryos per plate.
*Significantly different from the control (0 ABA) according to Dunnett's at the 0.05 level; ns = nonsignificant.

Table 4. Effect of MGBG on the growth and development of somatic embryogenic cultures of 'Thompson Seedless' grape.

MGBG conc (mM)	Culture fresh wt (mg) 1 mo.	Culture fresh wt (mg) 2 mo.	Culture fresh wt (mg) 3 mo.	Culture dry wt (mg) 3 mo.	No. cotyledonary embryos 1 mo.	No. cotyledonary embryos 2 mo.	No. cotyledonary embryos 3 mo.
0	1809	1673 ns	1676	374	3.0	2.6	3.3
0.1	1769 ns	1844 ns	1946 ns	439 *	4.0 ns	4.4 ns	3.4 ns
1	1528 ns	1429 *	471 *	190 *	5.2 ns	0.8 ns	0.4 *
10	850 ns	319 *	0 *	0	0 ns	0 ns	0 *

[z]Number of cotyledonary stage empbryos resembling zygotic embryos.
*Significantly different from the control (0 MGBG) according to Dunnett's at the 0.05 level; ns = nonsignificant.

the death of cells incubated on EMM with 10 mM MGBG by the third month. The production of normal grape somatic embryos was not affected by MGBG during the first 2 months of culture. However, the formation of cotyledonary stage embryos was inhibited after 3 months by 1 to 10 mM MGBG. No difference in the number of high quality cotyledonary embryos was detected among embryogenic cultures incubated on EMM with 0 or 0.1 mM MGBG throughout the course of the experiment.

Previous studies have suggested that the production of abnormal grape somatic embryos may be related to an increase in endogenous polyamine synthesis during the torpedo stage of development (Faure et al., 1991). MGBG inhibits the synthesis of two polyamines, spermidine and spermine, by blocking the decarboxylation of S-adenosylmethionine (Galston and Kaur-Sawhney, 1987). Polyamine and ethylene synthesis are interrelated, i.e., ethylene production elevates when polyamine synthesis is blocked and decreases when it is not (Galston and Kaur-Sawhney, 1987). MGBG has been shown to increase ethylene synthesis while inhibiting polyamine production (Roberts et al., 1984). Therefore, we do not know at present if the growth inhibition of grape embryogenic cells was caused by the addition of MGBG or by a possible increase in endogenous ethylene production.

This study demonstrates that grape somatic embryogenesis can be controlled using sucrose, ABA, and a specific polyamine biosynthesis inhibitor. The production of high quality somatic embryos was promoted by doubling the sucrose concentration of the standard medium currently used for grape somatic embryogenesis. Adding low concentrations (1 μM) of ABA improved plantlet formation from torpedo stage embryos but was ineffective in controlling precocious germination and secondary embryo formation. Further studies are required in order to identify an ABA concentration that controls precocious germination and secondary embryo formation without inhibiting plantlet formation. MGBG inhibited the growth and development of grape somatic embryos at concentrations above 0.1 mM but failed to improve the quality of grape somatic embryos. This suggests that polyamines may play a role in regulating the growth of grape somatic embryogenic cultures. Further investigation in the area of endogenous polyamine production may reveal inhibitors that promote the production of high quality grape somatic embryos without inhibiting embryo growth and development.

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