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Effects of sucrose and methylglyoxal bis-(guanylhydrazone) on controlling grape somatic embryogenesis

by

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S u m m a r y : The effects of sucrose and methylglyoxal bis-(guanylhydrazone) (MGBG) on grape (*Vitis vinifera* L. cv. Thompson Seedless) somatic embryogenesis was examined by subculturing somatic embryos and embryogenic cells monthly to embryo maintenance medium (MMS) containing 60, 90, 120, 150, or 180 g/l sucrose; or 0, 0.1, 1, or 10 mM MGBG for three months. The growth and development of grape embryogenic cultures was inhibited by incubating them on MMS with 150 or 180 g/l sucrose compared to 60, 90, or 120 g/l. Culture dry weight was significantly greater for embryogenic cells grown on MMS with 90 or 120 g/l sucrose compared with those reared on standard MMS (60 g/l sucrose), indicating that embryogenic cells grew better on MMS with 90 or 120 g/l sucrose and were less hydrated. The number of cotyledonary-stage somatic embryos that resembled zygotic embryos was improved 10.8- to 21.3-fold by incubating grape embryogenic cells on MMS with 90 or 120 g/l sucrose, respectively. Germination and plant development of grape somatic embryos was improved following incubation on MMS with 150 g/l sucrose before transfer to germination medium with benzyladenine. However, fewer embryos were produced on this medium compared to all other sucrose levels, suggesting that maintaining embryogenic cultures on MMS with 120 g/l sucrose followed by one transfer onto MMS with 150 g/l sucrose may improve embryo development and plant regeneration. MGBG at 1 to 10 mM inhibited the growth and development of grape embryogenic cultures. Exposure of embryogenic cells to 10 mM MGBG inhibited their growth and development through the course of the experiment and caused their death by the third month of culture. In contrast, a 3-month exposure was required to inhibit embryo growth in the presence of 1 mM MGBG. Addition of MGBG to MMS did not improve embryo quality or plant development.

K e y w o r d s : Vitis, polyamines, synthetic seeds, tissue culture.

A b b r e v i a t i o n s : ABA - abscisic acid, BA - benzyladenine, 2,4-D - 2,4-dichlorophenoxyacetic acid, MGBG - methylglyoxal bis-(guanylhydrazone), MMS - embryo maintenance medium

Introduction

Somatic embryos have been used for clonal propagation of elite lines and as "synthetic seeds" by encapsulating them in alginate or fluid-drilling gel, dehydrating them naked, or dehydrating embryos following encapsulation (GRAY and PUROHIT 1991 a, b; GRAY *et al.* 1995). Plants have been obtained from dehydrated somatic embryos of alfalfa [*Medicago sativa* L. (ANANDARAJAH and MCKERSIE 1990 b; SENARATNA *et al.* 1989)], grape [*Vitis* sp. (GRAY 1987, 1989)], maize [*Zea mays* L. (COMPTON *et al.* 1992)] and orchardgrass [*Dactylis glomerata* L. (GRAY *et al.* 1987)]. Synthetic seeds of grape might be used for germplasm conservation (GRAY and COMPTON 1993). Grape germplasm is currently maintained in vineyards, which exposes accessions to insects and diseases, and possible destruction by natural disasters. A synthetic seed system would allow grape accessions to be stored in seed repositories as clonal somatic embryogenic cultures or dehydrated, quiescent somatic embryos, eliminating the need for extensive germplasm collections in vineyards. In addition to these potential applications, embryogenic cultures recently have enabled the genetic transformation of *Vitis vinifera* L. grape (GRAY 1995).

An efficient embryo-to-plant regeneration system must be in place in order to utilize embryogenic culture systems. However, plant regeneration from grape somatic embryos is often difficult. Dehydration of grape somatic embryos improved plant formation (GRAY 1987, 1989), but the percentage of embryos that formed plants was low (~30 %). Plant regeneration of alfalfa (ANANDARAJAH and MCKERSIE 1990 a, b; SENARATNA *et al.* 1989) and maize (GREEN *et al.* 1983; PETERSEN *et al.* 1992) was improved by culturing somatic embryos on medium with high osmoticum. Transfer of somatic embryos to medium with high osmotica causes partial dehydration of cells within somatic embryos, which improves the production of storage compounds (starch, oils, lipids, etc.) and results in improved embryo quality and plant development (ANANDARAJAH and MCKERSIE 1990 a, b). In grape, the production of high quality somatic embryos is low. Subculturing grape somatic embryos to medium with high osmoticum may improve embryo quality and subsequent plant development.

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Elevated endogenous polyamine production has been implicated as a possible cause of abnormal somatic embryo development in grape (FAURE *et al.* 1991). The occurrence of abnormal grape somatic embryos coincided with an increase in endogenous polyamine production at the torpedo stage of embryo development. MGBG is a polyamine biosynthesis inhibitor that blocks spermidine and spermine synthesis by inhibiting the decarboxylation of S-adenosylmethionine (GALSTON and KAUR-SAWHNEY 1987). Addition of polyamine biosynthesis inhibitors, such as MGBG, to standard embryogenesis medium may improve development of normal embryos and improve plant formation by blocking endogenous polyamine production by somatic embryos. However, to understand the effects of sucrose and MGBG on globular-to-late stage embryo development, the growth of the subtending culture tissue, which in our grape system is composed wholly of embryogenic cells and young multicellular proembryos (GRAY 1992, 1995), also must be considered. The objective of this work was to examine the effects of sucrose 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 grape embryogenic cultures: Embryogenic cultures of 'Thompson Seedless' grape were established using a method adapted from GRAY (1989) and GRAY and MORTENSEN (1987). Leaf explants (1-5 mm) were excised from shoot-tip cultures maintained on modified C_2D medium (CHEE *et al.* 1984) with 5 µM BA (Sigma Chemical Co., St. Louis, MO) and incubated in 100 x 15 mm petri plates containing 25 ml of somatic embryo induction medium [NITSCH and NITSCH (1969) with (per liter) 20 g sucrose, 0.1 g myo-inositol, 1 µM BA, 5 µM 2,4-D, 10 µM ABA, and 7 g TC agar (JRH Biosciences, Lenexa, KS) at pH 5.5] for 6 weeks in the dark at 25 °C. Embryogenic cells, callus, and somatic embryos were transferred to 100 x 15 mm petri plates containing 50 ml of MMS [modified Murashige and Skoog medium (1962) with (per liter) 412.5 mg NH_4NO_3, 475 mg KNO_3 (instead of 1650 mg and 1900 mg, respectively), 1 g myo-inositol, 60 g sucrose, 2 g washed activated charcoal, and 7 g TC agar at pH 5.4] and maintained at 25 °C and low light intensity (0.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from cool-white fluorescent lamps and 16 h photoperiod). Embryogenic callus and cells were subcultured to fresh MMS monthly.

Embryogenic cultures consisting of 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. Cultures were at least 6 months old when used. Five plates were cultured per treatment, each containing 5 clumps of embryogenic material weighing approximately 15 mg (75 mg/plate). Treatments for all experiments were arranged in a completely randomized design. Culture fresh weight and the number of cotyledonary embryos that resembled zygotic embryos were recorded each month for 3 months. Culture dry weight was measured at the end of the experiment. Cultures were dried in an oven at 70 °C for 72 h. Experimental design followed that set forth for plant tissue culture experiments by COMPTON (1994). Statistical analysis was conducted using the GLM procedure of the statistical analysis system (SAS 1988). Treatment means were compared using regression and lack-of-fit (LOF) analysis. Polynomial contrasts statements were used to detect response trends in experiments with unevenly spaced treatments or unequal replication. In these cases contrast coefficients and regression models were calculated as described by CARMER and SEIF (1963). Percentage data were analyzed using Maximum Likelihood Analysis of Variance and means compared using standard error of the mean. In cases where treatment significance was observed but regression and LOF analyses failed to detect a trend, treatment comparisons were made with the experimental control (DUNNETT 1955).

Effects of sucrose on grape somatic embryogenesis and plant formation: The effects of sucrose on grape somatic embryogenesis was studied by subculturing embryogenic cells and somatic embryos to MMS containing 60, 90, 120, 150, or 180 g/l sucrose. Embryogenic cultures were subcultured monthly for 3 months. The effects of sucrose on plant development from somatic embryos was assessed by transferring cotyledonary stage embryos from each sucrose treatment to MSB (GRAY 1992). The number of embryos forming plants was recorded after 2 months on MSB.

The effects of MGBG on grape somatic embryogenesis were examined by subculturing embryogenic cells cultured on MMS-12%S (MMS with 120 g/l sucrose) to similar medium with 0, 0.1, 1, or 10 mM MGBG. The polyamine inhibitor was filter-sterilized and added to cooled, autoclaved medium.

Results and discussion

Effects of sucrose on grape somatic embryogenesis and plant development: There was a significant interaction between the medium sucrose concentration and time for culture fresh weight ($P < 0.001$). A decline in fresh weight gain was observed between the first and second subculture period when embryogenic cultures were incubated on medium with 60 g/l sucrose (Fig. 1). However, little difference was detected between the second and third subculture ($4760.2 - 2455.32x + 505.84x^2$; $r^2 = 0.6425$). No change in fresh weight gain was observed among embryogenic cells reared on medium with 150 g/l sucrose from the first to the third subculture ($864.79 - 55.21x$; $r^2 = 0.2590$). Fresh weight gain improved from the first to third subculture when embryogenic cells were grown on medium with 180 g/l sucrose ($829.695 - 299.58x + 83.65x^2$; $r^2 = 0.4516$). Fresh weight gain was similar for the first, second, and third subculture periods when grape embryogenic cells and embryos were grown on medium with 90 or 120 g/l sucrose (non-significant regression).

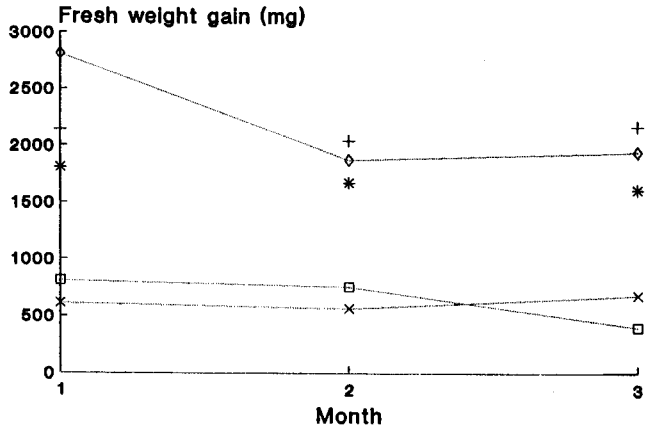

Fig. 1: Effect of sucrose and time in culture on fresh weight gain of grape somatic embryogenic cultures. Embryogenic cells were cultured on MMS with 60 ( ), 90 (+), 120 (*), 150 (□), or 180 (x) g/l sucrose for 3 months. Dotted line represents the predicted regression equation for each sucrose level. Each plate contained 75 mg of embryogenic material at the beginning of the experiment. A similar amount was subcultured to fresh medium each month.

Dry weight of embryogenic cultures was significantly influenced by the sucrose concentration in the medium ($P < 0.001$). Embryogenic cultures maintained on MMS with 90 or 120 g/l sucrose had the greatest dry weights (Fig. 2). Embryos grown on standard MMS (60 g/l), or MMS with 150 or 180 g/l sucrose had reduced dry weights. The response trend of embryogenic cultures to different sucrose concentrations best fit the cubic regression model ($-1774.06 + 57.92x - 0.4837x^2 + 0.001x^3$; $r^2 = 0.8815$). The moisture content of grape somatic embryos grown on MMS with 60, 90, 120, 150, and 180 g/l sucrose was 88, 77, 77, 73, and 72 %, respectively (data not shown), suggesting that grape somatic embryos grown on MMS with high sucrose contained less unbound water than similar embryos grown on standard MMS.

The number of high-quality somatic embryos was significantly affected by the sucrose concentration ($P < 0.001$) and length of exposure to the treatments ($P < 0.05$). However, there was no interaction between the two. The number

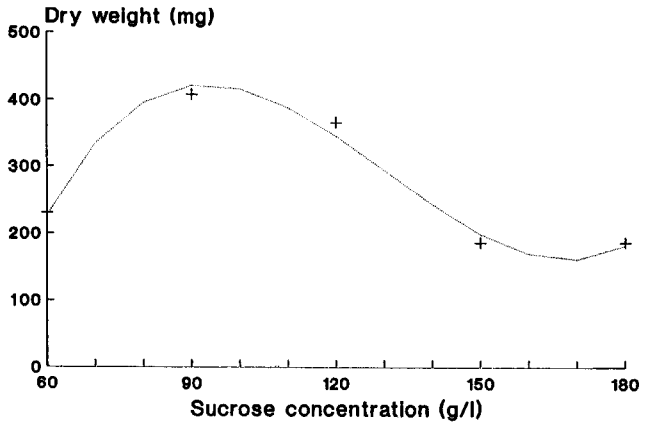

Fig. 2: Effect of sucrose concentration on dry weight of grape somatic embryogenic cultures. Dotted line represents the predicted regression equation. Treatment means are represented by +.

Table 1

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

Sucrose concentration (g/l)	Number of cotyledonary embryos: Mean[1]		Change from control[2]
60	0.4		
90	4.3	*	10.8
120	8.5	*	21.3
150	2.7	ns	6.8
180	0	ns	-

[1] Significance from the control (0) was determined using Dunnett's at the 0.05 (*) level; ns = nonsignificant.
[2] Obtained by dividing treatment means by the control mean.

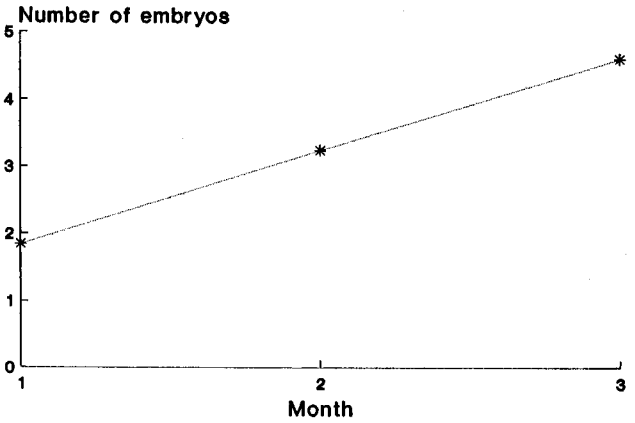

Fig. 3: Effect of length of time *in vitro* on the production of zygotic appearing grape somatic embryos. Dotted line represents the predicted regression equation. Treatment means are represented by *

of high-quality, cotyledonary somatic embryos was improved 10.8- and 21.3-fold over the control (60 g/l) when embryogenic cultures were incubated on medium with 90 or 120 g/l sucrose (Tab. 1). The number of embryos produced per plate increased linearly (0.4499 + 1.393x) between the first and third subcultures (Fig. 3), representing a 2.5-fold increase between the first and third subculture. Improvement in quality of embryos reared on MMS with 120 g/l 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 1990 a, b; PETERSEN *et al.* 1992).

The percentage of somatic embryos that formed plants was highest when they were reared on medium with 150 g/l sucrose (Tab. 2). Germination and plant development rates were 9.7-, 2.9-, and 5.6-fold lower among embryos grown on MMS with 60, 90, or 120 g/l sucrose, respectively. However, grape embryogenic cultures grew poorly when incubated on MMS with 150 g/l sucrose, the medium from

Table 2

Effect of sucrose concentration in MMS on germination of grape somatic embryos[1]

Sucrose (g/l)	Number of embryos[2]	Number of plants	Germination and plant development (%)
60	29	2	6.9 ± 4.7
90	30	7	23.3 ± 7.7
120	50	6	12.0 ± 4.6
150	6	4	66.7 ± 19.2

[1] Embryos were germinated on medium with 1 µM BA.
[2] Number of somatic embryos transferred to germination medium.

which embryos that readily germinated into plants were obtained. More high-quality cotyledonary stage embryos were obtained from embryogenic cultures maintained on MMS with 90 or 120 g/l sucrose, suggesting that grape embryogenic cultures should be maintained on medium with 90 or 120 g/l sucrose and transferred to medium with 150 g/l sucrose before transferring embryos to MSB germination medium.

The observed effects of sucrose on embryogenesis may be due to two factors, either alone or in combination. Sucrose may exert an osmotic effect, causing a lower intra- and intercellular embryo water content, mimicking zygotic embryogenesis and thereby promoting more normal development. Alternatively, increased sucrose may provide a necessary increase in carbon nutrition, allowing more storage compounds to be produced and, again, leading to more normal development (GRAY 1991 b).

Effects of MGBG on grape somatic embryogenesis: Addition of MGBG to MMS-12%S influenced the growth of grape embryogenic cultures ($P < 0.001$). However, there was a significant interaction between the MGBG concentration and length of time that embryogenic cultures were exposed to the polyamine biosynthesis inhibitor ($P < 0.001$). Addition of 10 mM MGBG to MMS-12%S caused a steady decline in fresh weight gain during the course of the experiment and resulted in the death of grape embryogenic cells by the third month ($1592.82 - 848.55x + 105.87x^2$; $r^2 = 0.9859$; Fig. 4). There was little difference in fresh weight gain between the first and second subculture when grape embryogenic cells were incubated on medium with 1 mM MGBG; however, a sharp decline in fresh weight gain was observed by the third month ($767.38 + 1189.74x - 429.48x^2$; $r^2 = 0.7663$). Addition of 0.1 mM MGBG failed to inhibit the growth of grape embryogenic cells compared to the controls, with the growth of embryogenic cells in both treatments remaining constant throughout the experiment (nonsignificant regression).

Addition of MGBG to MMS-12%S influenced culture dry weight ($P < 0.001$). Little difference in culture dry weight was detected between the 0, 0.1, and 1 mM treatments (Fig. 5). However, a significant decline in dry weight was observed at 10 mM MGBG. Polynomial contrast indicated that this response best fit the quadratic model ($427.79 - 25.46x + 0.2118x^2$).

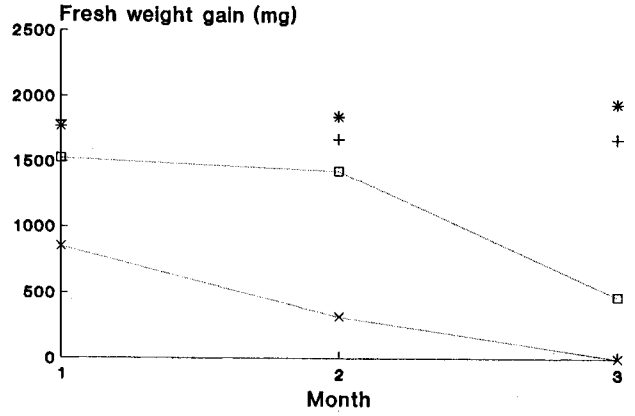

Fig. 4: Effect of MGBG concentration and length of exposure on fresh weight gain of grape somatic embryogenic cultures. Embryogenic cells were cultured on MMS with 0 (*), 0.1 (+), 1 (□), or 10 (x) mM MGBG for 3 months. Dotted line represents the predicted regression equation for each MGBG level. Each plate contained 75 mg of embryogenic material at the beginning of the experiment. A similar amount was subcultured to fresh medium each month.

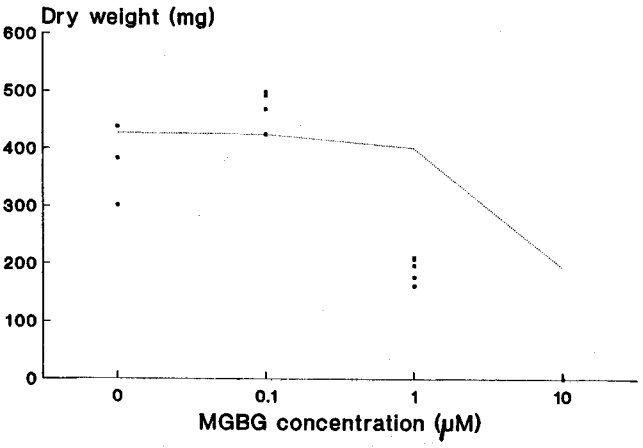

Fig. 5: Effect of MGBG concentration on dry weight of grape embryogenic cultures exposed to the polyamine inhibitor for 3 months. Dotted lines represent the predicted regression equation. Treatment data values are represented by ■.

The number of cotyledonary embryos per plate was affected by the addition of MGBG to MMS-12%S ($P < 0.001$). Embryo development was completely suppressed when grape embryogenic cells were incubated on MMS-12%S with 10 mM MGBG (Tab. 3). While there was no significant difference in embryo production in the presence of 0.1 or 1 mM MGBG; however, numerically, the 0.1 mM level resulted in a 40 % increase. This suggests that an expanded evaluation of low MGBG levels might be useful, despite the nonsignificant result of the present experiment. Due to the magnitude of potential positive response, this MGBG level should be re-evaluated to determine whether the lack of statistical significance was due excessive experimental variation.

The inhibition of embryogenic cell cultures suggests that polyamines may play an active role in controlling growth and development from the earliest stages of grape somatic embryogenesis. Previous studies suggest 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. MGBG has been shown to increase ethylene synthesis while inhibiting polyamine production (ROBERTS *et al.* 1984). Therefore, it is not clear if the growth reduction of grape embryogenic cells was caused by the inhibition of endogenous polyamine biosynthesis by MGBG or by an increase in endogenous ethylene production.

Table 3

Effect of MGBG on the growth and development of somatic embryogenic cultures of cv. Thompson Seedless grape

MGBG (mM)	Number of cotyledonary embryos Mean[1]		Change from control[2]
0	2.9		
0.1	4.2	ns	1.5
1.0	2.1	ns	0.7
10.0	0	*	-

[1] Significance from the control (0) was determined using Dunnett's at the 0.05 (*) level; ns = nonsignificant.
[2] Obtained by dividing treatment means by the control mean.

This study demonstrates that grape somatic embryogenesis can be controlled using sucrose and MGBG. Increasing levels of both factors inhibited the growth of undifferentiated embryogenic cells and multicellular proembryos, as measured by fresh and dry weight determination. The production of high-quality somatic embryos was promoted by doubling the sucrose concentration of the standard embryo maintenance medium currently used for grape somatic embryogenesis. MGBG inhibited the growth and development of grape somatic embryos at concentrations above 1 mM but failed to improve the quality of grape somatic embryos. This suggests that spermidine and spermine, the two polyamines inhibited by MGBG, may play a role in regulating the growth of grape somatic embryogenic cultures but not the production of high-quality somatic embryos. 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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References

ANANDARAJAH, T.; MCKERSIE, B. D.; 1990 a: Enhanced vigor of dry somatic embryos of *Medicago sativa* L. with increased sucrose. Plant Sci. **71**, 261-266.

- -; - -; 1990 b: Manipulating the desiccation tolerance and vigor of dry somatic embryos of *Medicago sativa* L. with sucrose, heat shock and abscisic acid. Plant Cell Rep. **9**, 451-455.

CARMER, S. G.; SEIF, R. D.; 1963: Calculation of orthogonal coefficients when treatments are unequally replicated and/or unequally spaced. Agron. J. **55**, 387-389.

CHEE, R.; POOL, R. M.; BUCHER, D; 1984: A method for large scale *in vitro* propagation of *Vitis*. New York Food Life Sci. Bull. **109**, 1-9.

COMPTON, M. E.; 1994: Statistical methods suitable for the analysis of plant tissue culture data. Plant Cell Tiss. Org. Cult. **37**, 217-242.

- -; BENTON, C. M.; GRAY, D. J.; SONGSTAD, D. D.; 1992: Plant recovery from maize somatic embryos subjected to controlled relative humidity dehydration. In Vitro Cell. Develop. Biol. **28 P**, 197-201.

DUNNETT, C. W.; 1955: A multiple comparisons procedure for comparing several treatments with a control. J. Amer. Stat. Assoc. **50**, 1096-1121.

FAURE, O.; MENGOLI, M.; NOUGAREDE, A.; BAGNI, N.; 1991: Polyamine pattern and biosynthesis in zygotic and somatic embryo stages of *Vitis vinifera*. J. Plant Physiol. **138**, 545-549.

GALSTON, A. W.; KAUR-SAWHENY, R.; 1987: Polyamines as endogenous growth regulators. In: DAVIES, P. J. (Ed.): Plant Hormones and their Role in Plant Growth and Development, 280-295. Martinus Nijhoff Publishers, Dordrecht.

GRAY, D. J.; 1987: Quiescence in monocotyledonous and dicotyledonous somatic embryos induced by dehydration. Proc. Symp. on Synthetic Seed Technology for the Mass Cloning of Crop Plants: Problems and Perspectives. HortScience **22**, 810-814.

- -; 1989: Effects of dehydration and exogenous growth regulators on dormancy, quiescence and germination of grape somatic embryos. In Vitro Cell. Develop. Biol. **25**, 1173-1178.

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

- -; 1995: Somatic embryogenesis in grape. In: JAIN, S. M.; GUPTA, P. K.; NEWTON, R. J. (Eds.): Somatic Embryogenesis in Woody Plants, 191-217. Kluwer Academic Publishers, Dordrecht.

- -; COMPTON, M. E.; 1993: Grape somatic embryo dormancy and quiescence: Potential of dehydrated synthetic seeds for germplasm conservation. In: REDENBAUGH, K. (Ed.): Synseeds: Applications of synthetic seeds to crop improvement, 367-379. CRC Press, Boca Raton, Fla.

- -; - -; HARRELL, R. C.; CANTLIFFE, D. J.; 1995: Somatic embryogenesis and the technology of synthetic seed. In: BAJAJ, Y. P. S. (Ed.): Biotechnology in Agriculture and Forestry, Vol. **30**, 126-151. Springer-Verlag, Heidelberg.

- -; CONGER, B. V.; SONGSTAD, D. D.; 1987: Desiccated quiescent somatic embryos of orchardgrass for use as synthetic seeds. In Vitro Cell. Develop. Biol. **23**, 29-33.

- -; MORTENSEN, J. A.; 1987: Initiation and maintenance of long term somatic embryogenesis from anthers and ovaries of *Vitis longii* 'Microsperma'. Plant Cell Tissue Org. Cult. **9**, 73-80.

- -; PUROHIT, A.; 1991 a: Quiescence and dormancy in somatic embryos. In: BAJAJ, Y. P. S. (Ed.): Biotechnology in Agriculture and Forestry, Vol. **17**, High-tech and Micropropagation I, 382-394. Springer-Verlag, Heidelberg.

- -; - -; 1991 b: Somatic embryogenesis and development of synthetic seed technology. Crit. Rev. Plant Sci. **10**, 33-61.

GREEN, C. E.; ARMSTRONG, C. L.; ANDERSON, P. C.; 1983: Somatic cell genetics systems in corn. In: FAZELAHMAD, A.; DOWNEY, K.; SCHULTZ, J.; VOELLMY, R.W. (Eds.): Advances in Gene Technology: Molecular Genetics of Plants and Animals. Miami Winter Symposium Series, Vol. **20**, 147-157. Academic Press, New York.

MURASHIGE, T.; SKOOG, F.; 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473-497.

NITSCH, J. P.; NITSCH, C.; 1969: Haploid plants from pollen grains. Science **163**, 85-87.

PETERSEN, W. L.; SULC, S.; ARMSTRONG, C. L.; 1992: Effect of nurse cultures on the production of macro-calli and fertile plants from maize embryogenic suspension culture protoplasts. Plant Cell Rep. **10**, 591-594.

ROBERTS, D. R.; WALKER, M. A.; THOMPSON, J. E.; DUMBROFF, E. B.; 1984: The effects of inhibitors of polyamine and ethylene biosynthesis on senescence, ethylene production and polyamine levels in cut carnation flowers. Plant Cell Physiol. **25**, 315-322.

SAS Institute, Inc.; 1988: SAS/STAT User's Guide. Release 6.03. SAS Institute, Inc., Cary, N.C.

SENARATNA, T.; MCKERSIE, B. D.; BOWLEY, S. R.; 1989: Desiccation tolerance of alfalfa (*Mediago sativa* L.) somatic embryos. Influence of abscisic acid, stress pretreatments and drying rates. Plant Sci. **65**, 253-259.

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