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Synchronized somatic embryo development in embryogenic suspensions of grapevine *Muscadinia rotundifolia* (Michx.) Small

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Summary

High-frequency, synchronous embryogenic systems in liquid culture facilitate plant regeneration and can be used as an essential model for performing functional genomics studies and understanding molecular aspect of the ontogenesis of higher plants. In the present study, synchronous somatic embryogenic cultures were developed for *Muscadinia rotundifolia* cv. Darlene and *Vitis vinifera* cv. Velika. High cell density and presence of 2,4-dichlorophenoxyacetic acid (2,4-D) proved to be essential for the establishment of the suspension cultures. Low cell density and continuous availability of auxin (2,4-D) was crucial for maintenance of suspension cultures. High cell density and withdrawal of 2,4-D is sufficient to advance somatic embryo development toward embryo differentiation and plantlets regeneration. Cells and cell clusters fractionation by density gradient centrifugation in Ficoll solution demonstrated to be a suitable method for separation of subpopulations with various potential for embryo development. The high frequency of synchronous development and differentiation of somatic embryos was attained essentially for the heaviest (at 16-18 % and >18 % Ficoll layer) cell population.

K e y w o r d s : somatic embryogenesis, *in vitro*, plant regeneration, grape, *Muscadinia*, *Vitis*.

Introduction

Plants are sessile organisms and in order to survive, grow and reproduce, they have developed strategies to circumvent environmental extremes and to take maximum advantage when conditions improve (Trewavas 1981; Smith and Krikorian 1990). This phenomenon, called phenotypic plasticity, includes instability in morphogenetic and developmental programs and the ability to respond to damaging external conditions. Somatic embryogenesis (SE) can be considered as an extreme state of phenotypic plasticity, where various developmental stages can be altered in the appropriate conditions (Dudits *et al*. 1995, Yeung 1995).

Muscadinia rotundifolia (Michaux) Small, the common muscadine grape, is native to the Southeastern United States and has been cultivated for more than 400 years (Olien 2001). The discovery of high volume anti-oxidant compounds in muscadine juice and wine has brought lately more attention to muscadine grape, not only as an alternative cash value crop for the Southeast, but a new healthy food as well (Colova 2005).

Notably cultivars of *Vitis vinifera* L. were among the first plants to be cultured *in vitro* (Morel 1944) and the grapevine *in vitro* regeneration has been lately studied extensively (reviews: Gray and Meredith 1992, Torregrosa 1995, Martinelli and Gribaudo 2001, Torregrosa *et al*. 2001). However, the successful initiation of somatic embryogenesis and plant regeneration remains unobtainable for many grape species and varieties.

Among responsive grape varieties, SE was most commonly reported from solid medium (SM) culture systems. Liquid medium (LM) embryogenic culture systems are relatively rare (Gray *et al.* 2005). Nevertheless, LM culture systems are preferable in several ways: they tend to proliferate much faster and with high efficiency than SM systems and plant regeneration frequencies are often better. Moreover, it has been reported that grape somatic embryos on solid medium showed signs of dormancy, whereas in suspension they were not dormant and demonstrated higher regeneration efficiency (Jayasankar *et al*. 2003).

The aim of the present study was to establish synchronous grapevine (*Muscadinia rotundifolia* and *Vitis vinifera*) somatic embryogenic cell lines in liquid culture with steady high conversion rate and regeneration capacity.

Material and Methods

P l a n t m a t e r i a l : Embryogenic calluses were obtained from *in vitro* leaf explants of *M. rotundifolia* 'Darlene' and *V. vinifera* 'Velika' (Colova *et al.* unpubl.). 'Velika' embryogenic cultures were established by Colova (Tsolova) in 1999 and served as a positive control for our experiments with *Muscadinia* embryogenic lines. Before suspension establishment, calluses were subcultured monthly for 6 x 45 d cycles on basal media solidified with 2.5 g l^{-1} gelan gum (Phytagel, Sigma) and supplemented with 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 µM 6-benzyladenine (BA). The basal media was half strength MS salts (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose; pH was adjusted to 6.1 with KOH and HCl before autoclaving at 120 ºC for 20 min.

C u l t u r e c o n d i t i o n s a n d e x p e r i m e n t a l p r o c e d u r e s : All suspension cultures were performed in Erlenmeyer flasks (125 ml), sealed with a double layer

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of aluminum foil and kept on gyratory shaker at 110 rpm, 26 ± 2 ºC, under continuous dark or 1.5 W m^{-2} photosynthetically active radiation (PAR), provided by 80 W cool-white fluorescent lamps. At least 5 flasks per treatment in three replications were used for each experiment. All quantitative data are presented as the mean value ± standard deviation.

Establishment and maintenance of suspension cultures: Samples of 1 or 2 g (fresh weight) of embryogenic callus were resuspended in 15 ml of the basal liquid medium supplemented with 5 µM 2,4-D (induction medium). After 1 week suspensions were diluted by adding equal amount of the same fresh medium. At day 14, all suspensions were filtered (stainless steel sieve, 2 mm pore size) to discard the largest aggregates. After centrifugation at 1000 x g for 5 min, the packed cell volume (PCV) was adjusted to 1 % (v/v) with fresh medium. Cultures were transferred to fresh medium every week by collecting the solid fraction on a nylon sieve (160 µm pore size) and keeping the initial cell inoculum at 1 % (w/v). At the end of each subculture, the fresh weight of the collected solid fraction was obtained in order to record suspension growth rate (final/initial fresh weight of cells and cell clusters).

Development and conversion of somatic embryos in liquid cultures: Suspension cultures grown for 6 months in maintenance medium were used for somatic embryo (SE) development and conversion in liquid culture. Two treatments, *i.e.* either a total absence of plant growth regulators (PGRs) or a combination of 1 µM BA and 0.5 µM l-naphthaleneacetic acid (NAA), were tested. The initial cell inoculum was adjusted to 2.5 % (w/v) for both treatments.

Synchronization of somatic embryo development: In order to synchronize SE development in liquid culture and to improve the efficiency, two procedures were tested. In the first, cell and cell clusters from 10 culture cycles on the liquid maintenance medium were separated into 4 size classes by 4 successive sievings through pore sizes: 160-500 µm, 500-1000 µm, 1000-2000 µm and >2000 µm. Aggregates of each class size were grown separately for 10 wk (10 weekly subcultures) in 25 ml of basal medium supplemented with 1 µM BA and 0.5 µM NAA (developmental medium). Initial cell inoculum was adjusted to 2.5 % (w/v). SE development was periodically monitored and the number of completely formed somatic embryos was recorded after 10 wk, at the end of the 10th subculture.

In the second procedure, the fraction ranging from 160-1000 µm was taken from cells cultured for 10 cycles on the liquid maintenance medium, grown for three subsequent cycles on PGR-free basal medium and separated by density gradient centrifugation in Ficoll solution (normally Ficoll solution contained 2 % sucrose) at 500 x g for 5 min. The density gradient was formed by 5 Ficoll concentrations gradually increasing from 10 % to 18 % by 2 %. After centrifugation, each cell fraction was re-suspended in the basal medium, centrifuged at 250 x g for 1 min to remove Ficoll residues and re-suspended again in 25 ml developmental medium with 2.5 % (w/v) as initial cell inoculum. The subculture on fresh medium was done after 1 wk without further centrifugation.

The number of somatic embryos formed from each fraction was recorded after 2 and 4 weeks of culture on the developmental medium.

Embryo conversion assay: Fresh weight of 10 g somatic embryos of the 18 % Ficoll fraction that were turning green were removed from liquid cultures and transferred to PGR-free solidified basal medium plated into 100 x 25 mm culture plates. The cultures were kept under 16 h-photoperiod culture conditions with 2.0 W m^{-2} PAR. Plant regeneration percentage rate was recorded within 45 d period.

Results

Establishment and maintenance of suspension cultures: Suspension cultures were successfully established when 2 g of proembryogenic callus (Fig. 1 A and B) were inoculated in 15 ml of liquid medium and then diluted with another 15 ml after 1 week. Examples of growth in fresh weight rates by percentage of increase are given in Tab. 1 with range from 141.4 to 195.5 with the larger increase occurring with the inoculum weight closer to 2 g. Under these conditions, after first filtration, cultures proliferated without forming large aggregates, and embryo development was mostly blocked at the proembryogenic mass (PEM) stage.

In comparison, a lower cell inoculum (1 g per 15 ml medium) at the beginning of suspension culture induced the formation of aggregates and sporadic germination of

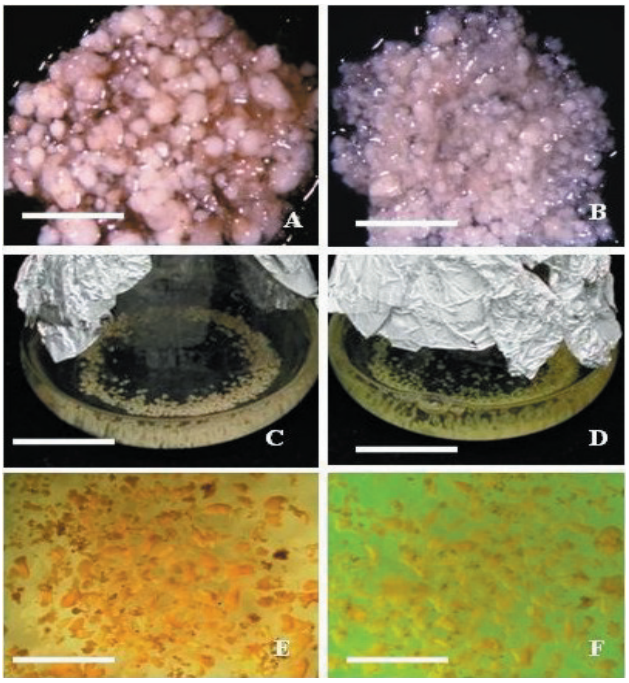

Fig. 1. Synchronized embryogenic lines of *M. rotundifolia* and *V. vinifera* grape: **A**) Embryogenic callus - 'Darlene'; **B**) Embryogenic callus - 'Velika'; **C**) Synchronized PEMs in suspension - 'Darlene'; **D**) Synchronized PEMs in suspension - 'Velika'; **E**) Differentiated SEs in suspension - 'Darlene'; **F**) Differentiated SEs in suspension - 'Velika'; (bar - 1 cm).

Table 1

'Percent of increase' of *Muscadinia* grape embryogenic lines (PEMs stage) for 1 week growth cycle in suspension.

Sample No.	Wo V + M (g)	FWb (g)	FWr (g)	% of increase	Significance
1	124	2.4	6.5	170.8	ns
2	123	2.2	6.5	195.5	**
3	115	2.4	6.9	187.5	ns
4	110	2.9	6.9	141.4	**
5	114	2.9	7.5	158.6	**

Wo: weight of the flask + media; FWb, FWr: fresh weight of the cell inoculum at the beginning and in the end of experiment.
*percentage of increase = FWr-FWb/FWb x100.
* Mean value of three replications ±SD.

somatic embryos formation.

The defined maintenance medium and cell inoculum (1 % w/v) demonstrated to be suitable for the maintenance of culture at the PEM stage (Fig. 1 C and D), with stable growth rate >100 % for at least of 6 months tested for *Muscadinia* grape.

Development of somatic embryos in liquid cultures: The lack of PGRs in the medium associated with high cell inoculum (2.5 % w/v) enabled PEM to advance to the 'globular' and 'torpedo' embryo stages. The SE differentiation occurred from PEM stage after 3 to 4 successive subcultures (Fig.1 E and F). Additional subculture under these conditions rarely resulted in further SE differentiation. In comparison, high cell inoculum in combination with the use of BA and NAA promoted embryo development and the presence of numerous SE completely formed after 4 weeks of culture can be observed. However, under these latter culture conditions, SE development occurred in a completely asynchronous manner and at low frequency (data not shown).

Synchronization of embryo development: Fractionation of cells and cell clusters on a size basis, followed by 2 subcultures at cell inoculum (2.5 % w/v) in the developmental medium, initially yielded homogenous cultures (Fig. 1 E and F). However, in both experiments on the developmental medium subsequently the somatic embryos started to mature asynchronously at the end of the third subculture regardless of size class of aggregates. Moreover, the SE were numerous, but often abnormal in morphology, *i.e.* cotyledon fusion or lacking cotyledons. No significant difference was observed among fractions with regard to growth rate during the course of subculture sequence (data not reported).

The fractionation by density gradient centrifugation in Ficoll solutions of cells and cell clusters previously grown for three cycles in the PGR-free medium enabled the cell population to be divided into two subpopulations in term of SE development capacity. Indeed, while no embryos were formed from cells of the lightest three fractions, a high frequency of embryo formation was observed in the two heaviest fractions, especially in the fraction collected in the 18 % Ficoll layer (Tab. 2). Even under these conditions, however, cells did not start differentiating embryos simultaneously. Some mature stages being already present after only one subculture. Nevertheless, at the end of the 4th week the homogeneity of mature embryos and their frequency were satisfactory.

Table 2

Number of differentiated *Muscadinia* SEs per replication in fractions obtained by Ficol density gradient centrifugation

Fraction (%)	Differentiated somatic embryos (number)	
	2 weeks	4 weeks
10-12	0	0
12-14	0	0
14-16	0	0
16-18	15.2 ± 2.4	81.1 ± 9.5
>18	20.7 ± 2.1	96.6 ± 10.2

* Mean value of three replications ± SD

Embryo conversion into plants: When plated on PGR-free solidified basal medium within 45 d, 79.6 % of the mature somatic embryos were converted into normal plantlets, 11.3 % showed abnormal morphology and 9.1 % continued to proliferate somatic embryos and did not develop at all (Fig. 2 B, C and D).

Discussion

Similar to many other woody *in vitro* plant regeneration systems, muscadine and *vinifera* grape somatic embryo development was arrested by 2.4-D and by high cell inoculum in the liquid initiation medium (Merkle *et al.* 1993). On the other hand, presence of auxin proved to be necessary for culture maintenance at the PEM stage by preventing SE differentiation. The withdrawal of PGRs

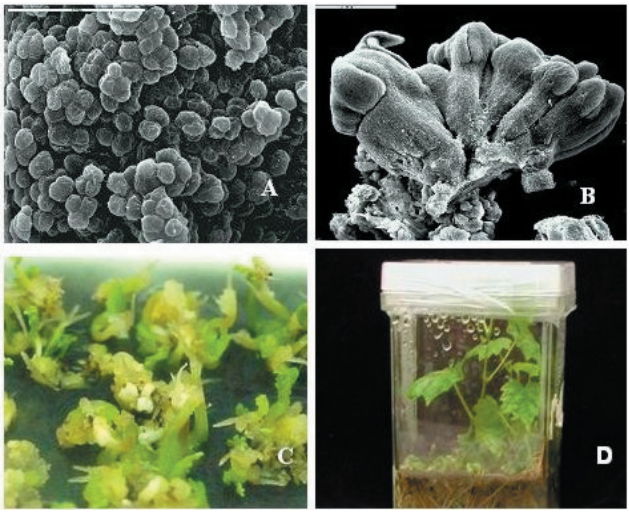

Fig. 2: Embryo conversion and plant regeneration of *M. rotundifolia* and *V. vinifera* somatic embryogenic lines: **A**) PEMs ('Velika') - scanning electron microscopy x 500.; **B**) SEs differentiation ('Velika') - scanning electron microscopy x 500; **C**) SEsgermination - 'Darlene'; **D**) Plant regeneration - 'Darlene'.

alone or addition of NAA and BA appears to be sufficient for progressing of the embryo development towards later stages of SE. A similar response was observed before by Coutos-Thevenot *et al*. (1992) and Jayasankar *et al.* (1999) in *vinifera* grape cultivars. However in those cases the withdrawal of 2,4-D alone was ineffective in promoting SE development beyond the 'heart' stage.

The fractionation of the cell population, through discontinuous density gradient centrifugation in Ficoll solutions, into two subpopulations with opposite SE development capacity supports the findings of Osuga and Komamine (1994) in *Daucus carota* and those of Jayasankar *et al.(*1999) in *Vitis vinifera*, where only the heaviest cytoplasm-rich cells proved to be suitable for embryo development. Occasionally observed browning of our *Muscadinia* cell lines (Fig. 1 A) has been reported also in *Mangifera indica* (Litz *et al.* 1995), and *Vitis vinifera* (Jayasankar *et al.* 1999) as related to high amounts of phenolic compounds.

However, the different response of the two fractions in terms of SE yield and culture synchronization might also be due to a beneficial effect of culture in PGR-free medium that was adapted only prior to fractionation by discontinuous density gradient centrifugation and not for separation on size basis. Certainly, while the fractionation via density gradient centrifugation was performed when the suspension was primarily at the globular embryo stage, the separation on size basis was done when the suspension was still at the PEM stage (Fig. 2 A).

In order to increase the percentage of conversion into normal plants, further studies are needed to improve this step of the protocol.

The results of the present study show that *Muscadinia rotundifolia* SE can be produced successfully in suspension culture with high efficiency by cell fractionation through Ficoll density gradient centrifugation and by manipulation of PGR and cell density in the liquid fraction. Taking into consideration the high regeneration rate, the present study is instrumental for high scale *in vitro* propagation and lays out the steps for large-scale *Muscadinia* somatic embryo production in bioreactors and further utilization for the various genetic enhancements. The fact that two cell populations can be distinguished with various potential in terms of somatic embryo development and differentiation could prove very useful for functional genomics studies of embryogenesis in woody perennial plants.

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