Somatic embryogenesis from immature zygotic embryos and monitoring the genetic fidelity of regenerated plants in grapevine

X.M. YANG*,**, L.Z. AN**, Y.C. XIONG**, J.P. ZHANG**, Y. LI** and S.J. XU**[1]

*Crop Institute, Gansu Academy of Agriculture Sciences, Lanzhou, 730070, P.R. China**
*School of Life Sciences, Lanzhou University, Lanzhou 730000, P.R. China***

Abstract

Somatic embryogenesis and plant regeneration were successfully established on Nitsch and Nitsch (NN) medium from immature zygotic embryos of six genotypes of grapevine (*Vitis vinifera*). The optimum hormone combinations were 1.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction and 1.0 mg dm^{-3} α-naphthalene acetic acid (NAA) + 0.5 mg dm^{-3} 6-benzyladenine (BA) for embryos production and 0.03 mg dm^{-3} NAA + 0.5 mg dm^{-3} BA for embryos conversion and plant regeneration. The frequency of somatic embryogenesis varied from 10.5 to 37.5 % among six genotypes and 15.5 - 42.1 % of somatic embryos converted into normal plantlets. The analysis of DNA content determined by flow cytometry and chromosome counting of the regenerated plantlets clearly indicated that no ploidy changes were induced during somatic embryogenesis and plant regeneration, the nuclear DNA content and ploidy levels of the regenerated plants were stable and homogeneous to those of the donor plants. RAPD markers were also used to evaluate the genetic fidelity of plants regenerated from somatic embryos. All RAPD profiles from regenerated plants were monomorphic and similar to those of the field grown donor plants. We conclude that somaclonal variation is almost absent in our grapevine plant regeneration system.

Additional key words: flow cytometry, plant regeneration, ploidy level, RAPD, *Vitis vinifera*.

Introduction

Research of genetic transformation in grapevine lagged compared to other crops since *in vitro* regeneration system is extremely difficult to establish. Most studies are focused on somatic embryogenesis (Kobayashi *et al*. 2002) because chimaeras occur frequently during genetic transformation (Iocco *et al.* 2001). Recently, some grapevines cells have been genetically transformed and plants regenerated using somatic embryogenic cultures as the original target tissue; most of them are *Vitis vinifera* cultivars (Kobayashi *et al*. 2002). However, due to high genotype dependence, some grape species and cultivars remain recalcitrant to the process of embryogenesis and transformation (Motoike *et al.* 2001). There have been some subsequent reports on somatic embryogenesis and plant regeneration corresponding to genotype, medium, hormone, concentration of sugar, organic component, *etc*. The frequency of somatic embryogenesis was commonly low. In addition, plantlets derived from *in vitro* culture might exhibit morphological, cytological, and molecular variations, which are often heritable. However, information about genetic fidelity of plants regenerated from somatic embryos in grapevine is rather scarce.

Therefore, it is very important to establish the safe system with high reproducibility and efficiencies in the most agronomical important genotypes suitable for future genetic transformation of grapevine. In the study reported here, we developed a safe *in vitro* somatic embryogenesis and plant regeneration system, and evaluated genetic fidelity of plants regenerated from somatic embryos.

Received 12 July 2006, *accepted* 24 March 2007.
Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - 6-benzyladenine; MS - Murashige and Skoog medium; NAA - α-naphthalene acetic acid; NN - Nitsch and Nitsch medium.
Acknowledgement: This research was financially supported by the National Natural Science Foundation of China (Nos. 90302010, 30570271) and the project of database platform construction for basis science and technology resource of the Ministry of Education of P.R. China (No. 505016).
[1] Corresponding author; fax: (+86) 931 8912561, e-mail: xushijian@lzu.edu.cn

Materials and methods

Plants and culture conditions: Following the protocol for immature zygotic embryos cultures reported by Emershad and Ramming (1994), the cluster of six diploid grapevine (*Vitis vinifera* L.) cultivars Cabernet France, Cabernet Sauvignon, Heijiamei, Merlot, Pinot Noir, and Sinsaut (all supplied by School of Life Science and Technology, Gansu Agricultural University, Lanzhou, China) were collected 45 - 50 d after pollination. Only large-seeded berries were selected and surface-sterilized for 5 min in a solution of 1.3 % sodium hypochlorite containing one drop of *Tween 20* and rinsed twice in sterile water. Large immature seeds were extracted from the fruit under sterile conditions, and immature zygotic embryos were excised from seeds and inoculated on Nitsch and Nitsch (1969; NN) medium supplemented with 1.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) for callus. The embryogenic calluses were subcultured every four-week on NN medium supplemented with 1.0 mg dm^{-3} α-naphthalene acetic acid (NAA) and 0.5 mg dm^{-3} 6-benzyladenine (BA) for somatic embryo production. Vigorously growing somatic embryos were selected and transferred to NN regeneration medium supplemented with 0.03 mg dm^{-3} NAA and 0.5 mg dm^{-3} BA for somatic embryo conversion and plant regeneration. The pH was adjusted to 6.0 and media were solidified with 7 g dm^{-3} agar prior to autoclaving at 121 °C for 15 min. For somatic embryo conversion, development and plantlet regeneration, the cultures were incubated in growth room at a temperature of 26 ± 2 °C with an irradiance of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps for 16-h photoperiod.

Flow cytometric analysis and cytological analysis: Flow cytometry was used for determination of ploidy stability. One young leaf from plants regenerated from somatic embryo belonging to cultivar Sinsaut was taken for identification of ploidy level by flow cytometry. Nuclear suspensions from leaves were prepared according to Galbraith *et al*. (1983). In brief, nuclei were extracted by chopping leaf tissue with a razor blade into a Petri dish containing 2 - 3 cm^3 ice-cold Galbraith's buffer with 3 % polyvinylpyrrolidone-10 to inhibit oxidation. The suspensions of released nuclei were filtered through 32-μm stainless steel mesh filter into a conical centrifuge tube and made up to about 15 cm^3 with ice-cold modified Galbraith's buffer before centrifugation at 300 *g* for 4 min. The supernatant was removed and the pellet resuspended in Galbraith's buffer. To label the DNA, we stained nuclei with propidium iodide. Nuclei were left to equilibrate for at least 30 min on ice before analysis. Nuclei were analyzed using *Facscan* laser flow cytometry (*Becton Dickinson*, USA) equipped with an argon-ion laser turned at wavelength of 448 nm. Prior to analysis, the gain of the flow cytometry was adjusted so that the peak corresponding to G_0/G_1 nuclei isolated from leaves of diploid grapevine plants was localized on channel 50. This calibration was checked periodically. Ploidy estimation was also determined by traditional chromosome counting. A total of 14 plantlets regenerated from somatic embryos belonging to cultivar Sinsaut were used for flow cytometric and cytological study in comparison with the field grown donor plants.

DNA extraction and PCR amplification: Plantlets that had previously been subjected to flow cytometric and cytological studies were subjected to RAPD analysis. DNA of 14 plants derived from somatic embryos belonging to cultivar Sinsaut and the field grown donor plants was extracted from young leaves following the method described by Hanania *et al.* (2004). In prescreen with 38 primers based on amplification of donor plant, eight arbitrary 10-base primers were selected for PCR amplification. Amplification reactions were performed with 0.025 cm^3 of 10× assay buffer, 2.0 of 1.25 mM each of dNTP's, 15 ng of the primer, 1 × Taq polymerase buffer, 0.5 units of Taq DNA polymerase (*TaKaRa*, China), 2.5 mM $MgCl_2$, and 30 ng of genomic DNA. DNA amplification was performed in a *Perkin Elmer Cetus 480* DNA thermal cycler programmed for 45 cycles as follows: 1st cycle of 3.5 min at 92 °C, 1 min at 35 °C, 2 min at 72 °C; followed by 44 cycles each of 1 min at 92 °C, 1 min at 35 °C, 2 min at 72 °C followed by one final extension cycle of 7 min at 72 °C. The amplification products were separated by electrophoresis in 1.2 % (m/v) agarose gels with 0.5× TBE buffer, stained with 0.2 mg dm^{-3} ethidium bromide. A 1 kb DNA ladder was used as molecular standards and the bands were visualized and analyzed by *JD-801 Gel Electrophoresis Image* analytic system (Jiangsu, China). All the reactions were repeated at least twice.

Results

Somatic embryogenesis: All six grapevine cultivars tested were capable of forming embryogenic callus from immature embryos (Table 1). During the first 2 weeks in culture, immature embryos of grapevine turned dark brown. However, soon after, the immature embryos resumed growth and produced brown semi-friable callus within 4 - 6 weeks on NN medium containing 1.0 mg dm^{-3} 2,4-D. When the callus was transferred to NN medium supplemented with 1.0 mg dm^{-3} NAA and 0.5 mg dm^{-3} BA, it developed yellow-white embryogenic tissue, and small translucent globular embryos were visible within 2 weeks. The establishment of embryogenic cultures was followed by the typical globular, pyriform, cordate, and cotyledonary stages of somatic embryos within 3 - 4

weeks. In the present study, somatic embryos appeared to be fused at the base and sometimes subculture caused them to recallus. Therefore it became necessary for the callus to be excised and subcultured along with the somatic embryos intact. Significant differences in callus induction and somatic embryogenesis were observed between cultivars indicating genotypic differences (Table 1). Amongst the six cultivars tested, Sinsaut, Pinot Noir and Cabernet France exhibited higher rate of somatic embryos production (37.5, 34.0, and 32.5 %, respectively) than other cultivars (Table 1).

Embryo conversion and plant regeneration: Germination of somatic embryos is characterized by cotyledon expansion and chlorophyll formation, followed by hypocotyl elongation. In the present study, embryos conversion and plants regeneration were achieved after about 50 d of cultivation of embryogenic cultures on NN

Table 1. Rate of embryogenic callus induction, somatic embryo production and plantlets regeneration of six grapevine genotypes from immature zygotic embryos on NN medium supplemented with 1.0 mg dm^{-3} 2,4-D for callus induction and 1.0 mg dm^{-3} NAA + 0.5 mg dm^{-3} BA for embryos production and 0.03 mg dm^{-3} NAA + 0.5 mg dm^{-3} BA for plant regeneration, respectively. Means ± SE, n = 3. Date within column followed by different letters differ significantly at 5 % level of probability using the Fisher test.

Grapevine genotypes	Number of inoculated embryos	Callus induction [%]	Somatic embryo production [%]	Plantlets regeneration [%]
Cabernet France	46	82.6 ± 1.8^{a}	32.5 ± 1.4^{a}	32.1 ± 0.8^{b}
Cabernet Sauvignon	38	65.8 ± 1.2^{b}	26.5 ± 0.9^{b}	18.5 ± 0.6^{d}
Heijiamei	48	85.4 ± 2.3^{a}	10.5 ± 0.4^{c}	15.5 ± 0.3^{d}
Merlot	35	74.3 ± 1.7^{a}	28.5 ± 1.5^{b}	23.3 ± 1.1^{c}
Pinot Noir	44	84.1 ± 2.1^{a}	34.0 ± 1.2^{a}	33.4 ± 1.4^{b}
Sinsaut	40	80.0 ± 2.4^{a}	37.5 ± 1.8^{a}	42.1 ± 1.5^{a}

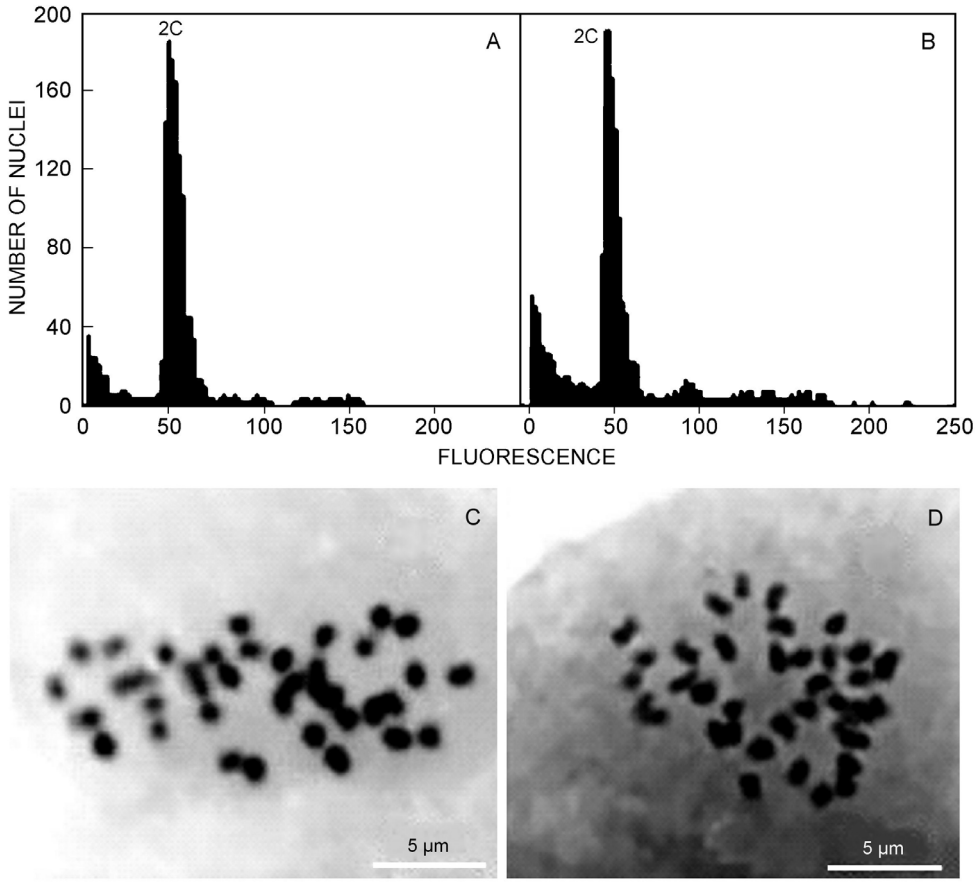

Fig. 1. DNA histograms of fluorescent intensity of nuclei showing 2C peaks and chromosomes with 2n=2x=38 on root tips of plants belonging to cultivar Sinsaut (*A*, *C* - field grown plant, *B*, *D* - plantlet regenerated from somatic embryos).

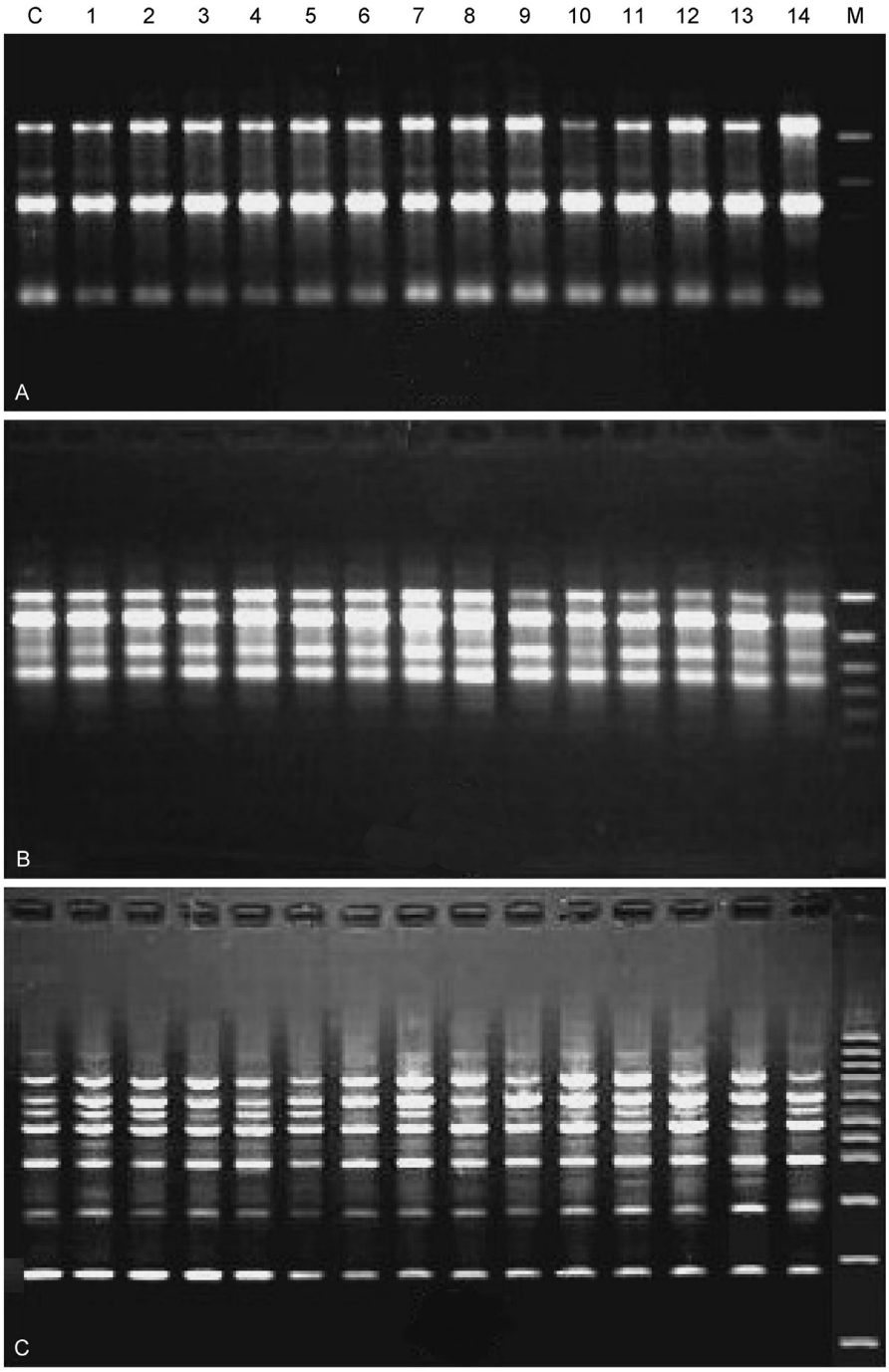

Fig. 2. RAPD banding patterns of donor mother plant (*lane C*) and different plantlet regenerated from somatic embryos (*lanes 1 - 14*) of cv. Sinsaut. The pattern generated by PCR amplification using the random primers S353 (*A*), S20 (*B*) and S37 (*C*). M - molecular mass markers (1 kb DNA ladder).

medium supplemented with 0.03 mg dm^{-3} NAA and 0.5 mg dm^{-3} BA. Differences in embryo conversion and plant regeneration of different genotypes have also been observed (Table 1). On average, 15.5 - 42.1 % somatic embryos successively converted and germinated into normal shoots, whereas others had cotyledons, but failed to convert and some produced secondary somatic embryos. In present study during the subculture on conversion medium, a large amount of secondary embryos was produced from single, mature deformed embryo. Although secondary embryogenesis is considered as a method for maintaining the regenerative ability of cultures, it may also hinder the conversion and normal development of somatic embryos. This might also account for the high incidence of underdeveloped embryos observed in this study. Therefore, further studies

are needed to optimize the percentage of somatic embryo conversion to plantlets. Further development and propagation of normal plantlets was established on Murashige and Skoog (1962; MS) proliferation medium containing 0.03 mg dm^{-3} NAA.

Analysis of the ploidy stability of regenerated plants: Flow cytometry performed on regenerated plant was employed to give an accurate estimation of nuclear DNA content. The most noteworthy observation is that plantlet regenerated from somatic embryo and donor plants showed the same ploidy level. The presence of one single peak in all the analyzed samples at channel 50 demonstrated the presence of homogenous 2C nuclei, without any detectable aneuploidy (Fig. 1*A*,*B*). The nuclear DNA content of the regenerated plants analyzed by flow cytometry was similar or close to that of the donor plants. The chromosome counts on root tips of 14 regenerated plants (cv. Sinsaut) also showed identical ploidy level to donor plant (2n=2x=38) (Fig. 1*C*,*D*).

Monitoring of genetic fidelity by RAPD: In order to further confirm genetic fidelity of plants regenerated from somatic embryos, the quality of 14 regenerated plants was screened with the 38 random RAPD primers. The results were scored as patterns of bands obtained from the regenerated plants and compared with the donor plants maintained in the field. Out of 38 random primers tested, eight primers that produced distinct amplification profiles, displayed same banding pattern in all the 14 plants as the DNA sample from donor plant. The total number of amplification products generated by polymerase chain reaction was 46 bands (5.8 bands per primer). The size of polymorphic fragments with eight primers varied from 150 bp in S20 to 1200 bp in S37 and the number of amplified products ranged from 3 in S20 to 7 in S37. From the representative profile of the 14 regenerated plants and the control with three primers (Fig. 2), it was obvious that the regenerated plants showed identical RAPD profiles (*i.e*. no polymorphism was observed). These results confirmed the genetic fidelity of the grapevine plants regenerated from somatic embryos.

Discussion

Somatic embryogenesis is the most direct way to regenerate plants from single cells or protoplasts. Somatic embryos or embryogenic cultures are also appropriate material for cryopreservation (Wang *et al*. 2004), development of artificial seeds or genetic transformation (Vicient and Martínez 1998). However, plants derived from *in vitro* culture might exhibit somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variation, which is a welcome source of genetic variation for crop breeding (Heinze *et al*. 1995), can pose a severe threat to the genetic fidelity of regenerated plants, which is particularly required during the genetic transformation experiments and to achieve genetic uniformity of the propagules (Bhatia *et al*. 2005). Somaclonal variation can either bring changes at the DNA level or it may induce changes in chromosome numbers. In general, morphological markers, chromosome analysis, isoenzyme or DNA markers may be used to detect somaclonal variation. In our study, three approaches, chromosome counting, flow cytometry and RAPD, were chosen to detect somaclonal variation. No genetic instabilities were detected among the somatic embryo-derived plants, and between the regenerated plants and the mother plants. As found in the present study, various investigators have observed the absence of variations. Loureiro *et al*. (2005) used flow cytometry to assess ploidy stability of somatic embryogenesis process in cork oak. No significant differences were detected among the somatic embryo-derived plants and the mother plants. Gesteira *et al*. (2002) used RAPD markers to evaluate genetic stability of soybean plants, obtained through somatic embryogenesis. RAPD analysis did not show gross somaclonal variation with respect to mother plants. In a similar way, several authors using flow cytometry, RAPD or RFLP also failed to observe somaclonal variations in various species (Pinto *et al*. 2004, Saker *et al.* 2005, Latto *et al.* 2006, Rady 2006, Loureiro *et al*. 2007).

In contrast, Hashmi *et al*. (1997) detected somaclonal variants in peach regenerants initiated from two different embryo callus cultures using RAPD. Kunitake *et al*. (1998) detected little ploidy and phenotypic variation among somaclones obtained through somatic embryogenesis from asparagus genotypes. Fourre *et al*. (1997) evaluated somaclonal variation in embryogenic clones of Norway spruce. Although variation was detected by cytogenetic and morphogenetic analyses, none was observed using RAPD analysis, in spite of using several primers. Similarly, Rani *et al*. (1995) found RAPD variations amongst 23 micropropagated *Populus deltoides* plants originating from the same somatic embryos. It is understood that many factors associated with culture manipulations can lead to the induction of genetic instability. In general, culture method and environment, species, explants type, concentration and type of plant growth regulators, are known to be associated with the occurrence of somaclonal variation (Rani and Raina 2000). Somaclonal variation is a very complex problem that needs several approaches to be correctly appreciated. It is essential to verify the clonal fidelity and field performance of somatic embryo-derived plants to ensure that somatic embryogenesis for each particular species is not causing aberrations.

In our study, the similarities in nuclear DNA content, chromosome counts and RAPD banding patterns in different plants derived from somatic embryos may suggest genetic fidelity and it therefore possible to postulate that tissue culture-derived grapevine plants are true-to-type. In summary, we have developed a general,

simple and safe system for somatic embryogenesis and plant regeneration of grapevine, which can be applied to important grapevine cultivars and represents a significant step towards the successful application of gene technology to improvement of established premium grapevine cultivars. Currently, we are developing *Agrobacterium*-mediated genetic transformation system based on plant regeneration via somatic embryogenesis in grapevine.

References

Bhatia, P., Ashwath, N., Senaratna, T., Krauss, S.L.: Genetic analysis of cotyledon derived regenerants of tomato using AFLP markers. - Curr. Sci. **88**: 280-284, 2005.

Emershad, R.L., Ramming, D.W.: Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). - Plant Cell Rep. **14**: 6-12, 1994.

Fourre, J.L., Berger, P., Niquet, L., Andre, P.: Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. - Theor. appl. Genet. **94**: 159-169, 1997.

Galbraith, D.H., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., Firoozabady, E.: Rapid flow cytometric analysis of the cell cycle in intact plant tissue. - Science **220**: 1049-1051, 1983.

Gesteira, A.S., Otoni, W.C., Barros, E.G., Moreira, M.A.: RAPD-based detection of genomic instability in soybean plants derived from somatic embryogenesis. - Plant Breed. **121**: 269-271, 2002.

Hanania, U., Velcheva, M., Sahar, N., Perl, A.: An improved method for isolating high-quality DNA from *Vitis vinifera* nuclei. - Plant mol. Biol. **22**: 173-177, 2004.

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L., Hammerschlag, F.: RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. - Plant Cell Rep. **16**: 624-627, 1997.

Heinze, B., Schmidt, J., Cassells, A.C., Jones, P.W.: Monitoring genetic fidelity vs. somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. - Euphytica **85**: 341-345, 1995.

Iocco, P., Franks, T., Thomas, M.R.: Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. - Transgenic Res. **10**: 105-112, 2001.

Kobayashi, S., Ishimaru, M., Hiraoka, K., Honda, C.: *Myb*-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. - Planta **215**: 924-933, 2002.

Kunitake, H., Nakashima, T., Mori, K., Tanaka, M.: Somaclonal and chromosomal effects of genotype, ploidy and culture duration in *Asparagus officinalis* L. - Euphytica **102**: 309-316, 1998.

Larkin, P.J., Scowcroft, W.R.: Somaclonal variation. A novel source of variability from cell cultures for plant improvement. - Theor. appl. Genet. **60**: 197-214, 1981.

Latto, S.K., Bamotra, S., Dhar, R.S., Khan, S., Dhar, A.K.: Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker – an endangered medicinal herb. - Plant Cell Rep. **25**: 499-506, 2006.

Loureiro, J., Capelo, A., Brito, G., Rodriguez, E., Silva, S., Pinto, G., Santos, C.: Micropropagation of *Juniperus phoenicea* from adult plant explants and analysis of ploidy stability using flow cytometry. - Biol. Plant. **51**: 7-14, 2007.

Loureiro, J., Pinto, G., Lopes, T., Doležel, C., Santos, C.: Assessment of ploidy stability of somatic embryogenesis process in *Quercus suber* L. using flow cytometry. - Planta **221**: 815-822, 2005.

Motoike, S.Y., Skirvin, R.M., Norton, M.A., Otterbacher, A.G.: Somatic embryogenesis and long term maintenance of embryogenic lines from fox grapes. - Plant Cell Tissue Organ Cult. **66**: 121-131, 2001.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

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

Pinto, G., Loureiro, J., Lopes, T., Santos, C.: Analysis of the genetic stability of Eucalyptus *globulus Labill* somatic embryos by flow cytometry. - Theor. appl. Genet. **109**: 580-587, 2004.

Rady, M.R.: *In vitro* culture of *Gypsophila paniculata* L. and random amplified polymorphic DNA analysis of the propagated plants. - Biol. Plant. **50**: 507-513, 2006.

Rani, V., Parida, A., Raina, S.N.: Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. - Plant Cell Rep. **14**: 459-462,1995.

Rani, V., Raina, S.N.: Genetic fidelity of organized meristem derived micropropagated plants: a critical reappraisal. - *In Vitro* cell dev. Biol. Plant. **36**: 319-330, 2000.

Saker, M.M., Adawy, S.S., Mohamed, A.A., El-Itriby, H.A.: Monitoring of cultivar identity RAPD in tissue culture-derived date palms using RAPD and AFLP analysis. - Biol. Plant. **50**: 198-204, 2005.

Vicient, C.M., Martínez, F.X.: The potential uses of somatic embryogenesis in agroforestry are not limited to synthetic seed technology. - Rev. Bras. Fisiol. Veg. **10**: 1-12, 1998.

Wang, Q.C., Mawassi, M., Sahar, N., Li, P., Violeta, C.T., Gafny, R., Sela, I., Tanne, E., Perl, A.: Cryopreservation of grapevine (*Vitis spp.*) embryogenic cell suspension by encapsulation-vitrification. - Plant Cell Tissue Organ Cult. **77**: 267-275, 2004.