Vitis **43** (4), 169–173 (2004)

Somatic embryogenesis of *Vitis vinifera* L. (cv. Sugraone) from stigma and style culture

C. Morgana[1), 2)], R. Di Lorenzo[2)] and F. Carimi[1)]

[1)] Istituto di Genetica Vegetale, Sezione di Palermo, CNR Corso Calatafimi 414, Palermo, Italia
[2)] Dipartimento di Colture Arboree, Università degli Studi di Palermo, Palermo, Italia

Summary

Somatic embryo and plant regeneration were induced from stigma and style culture of grapevine (*Vitis vinifera* L. cv. Sugraone). To obtain somatic embryogenesis, explants were cultured on Nitsch and Nitsch basal medium (NN) supplemented with 88 mM sucrose and various combinations of the auxin β-naphthoxyacetic acid (NOA, 0-10 µM) and the cytokinin 6-benzylaminopurine (BA, 0-9 µM). Growth regulators (BA and NOA) in the culture medium were essential for induction of somatic embryogenesis since explants incubated on hormone-free medium never regenerated somatic embryos. Usually, the regenerated somatic embryos become visible as small white globular structures on the surface of the callus 3-4 months after culture initiation. The best embryogenic response (27 %) was obtained when stigma and style explants were cultured on NN medium supplemented with 5 µM NOA and 9 µM BA. Somatic embryos developed into plantlets when transferred to a hormone-free semisolid NN medium. 35 % of primary somatic embryos showed secondary embryogenesis.

K e y w o r d s : Anthers, grapevine, plant regeneration, proembryogenic masses, *Vitis vinifera.*

A b b r e v i a t i o n s : BA: 6-benzylaminopurine; NN: Nitsch and Nitsch medium; NOA: β-naphthoxyacetic acid; PEMs: proembryogenic masses.

Introduction

Genetic improvement of grapevine by conventional breeding is impeded by long juvenile periods and inbreeding depression (Jayasankar *et al.* 2000). Therefore alternative approaches involving the use of somatic embryogenesis would be of great benefit for genetic improvement programs and virus elimination (Goussard *et al.* 1991, Goussard and Wiid 1992, Bouquet and Torregrosa 2003).

Reliable protocols for the regeneration of *Vitis vinifera* L. somatic embryos have been reported in literature (Martinelli and Gribaudo 2001). Somatic embryos have been regenerated from different explant types such as unfertilized ovules, anthers, immature zygotic embryos, immature leaves, tendrils, immature ovaries, leaf discs and filaments (Mullins and Srinivasan 1976, Mauro *et al.* 1986, Stamp and Meredith 1988 a, b, Salunkhe *et al.* 1997, Nakano *et al.* 2000, Das *et al.* 2002, Nakajima and Matsuta 2003). Although several types of tissues have been successfully used, anthers are considered the most suitable explant for the regeneration of somatic embryos; therefore, they are currently used to induce the regeneration of somatic embryos in many genotypes (Bouquet and Torregrosa 2003). The number of genotypes from which somatic embryogenesis can be induced is increasing but still limited (Nakajima and Matsuta 2003). Therefore new efficient protocols involving the use of alternative explants for the induction of somatic embryogenesis offer a highly attractive system for physiological and genetic studies.

In the present work, a typical explant (anthers) used in previous work for the regeneration of *V. vinifera* somatic embryos, and alternatively floral tissues (stigmas and styles) were compared in order to improve the regeneration of plants through somatic embryogenesis.

Material and Methods

P l a n t m a t e r i a l : Cultures were initiated from anthers and stigma-style explants. Immature flowers from field-grown plants of *V. vinifera* cv. Sugraone were used. Flowers length: 1.5 - 2.5 mm were collected when the petals were still closed (about 1 week before flowering). They were surface-sterilised with 75 % ethanol-water for 3 min and 0.6 % sodium hypochlorite and 0.05 % Tween 20 (Sigma) for 15 min. Flowers were then rinsed three times in sterile distilled water for 5 min. Each corolla was removed under sterile conditions using a stereo microscope. Anthers and stigma-style explants were excised with a sterile forceps and sharp blade from the flowers and placed in the petri dishes containing the different media. Stigma-style explants were placed vertically onto plastic Petri dishes with the cut surface in contact with the medium. Five explants were placed in each Petri dish and 50 explants were used per treatment.

M e d i a a n d c u l t u r e c o n d i t i o n s : In all the experiments the culture medium consisted of Nitsch and Nitsch (1969) (NN) basal salts and vitamins with the addition of 88 mM sucrose. Three groups of experiments were carried out to compare the embryogenic potential of anther and stigma-style explants. The first set of experiments was carried out to compare culture media reported in literature

Correspondence to: Dr. F. Carimi, Istituto di Genetica Vegetale, Consiglio Nazionale delle Ricerche, CNR Corso Calatafimi 414, 90128 Palermo, Italy. Fax: +39-49-8276300. E-mail: carimi@civ.bio.unipd.it

for the regeneration of somatic embryos in 4 species of the genus *Vitis* (GRAY 1992, MARTINELLI *et al.* 1993, TSOLOVA and ATANASSOV 1994, HARST 1995). The 4 different hormonal combinations to test the embryogenic potential of stigma-style explants are presented in Tab. 1. Explants incubated without growth regulators were used as control. In a second set of experiments 16 different growth regulator combinations were used: the auxin β-naphthoxyacetic acid (NOA, Sigma N-3019, 0, 1, 5 and 10 μM) associated with the cytokinin 6-benzylaminopurine (BA, Sigma B-3274, 0.0, 0.5, 4.5 and 9.0 μM). In a third set of experiments stigma and style explants were collected during the third year and incubated only on 9 μM BA + 5 μM NOA or 9 μM BA + 10 μM NOA supplemented media.

After the addition of growth regulators the pH of the medium was adjusted to 5.6 before autoclaving at 121 °C for 20 min. All the media were gelled with 0.8 % (w/v) agar. Plastic Petri dishes, 55 mm in diameter and 15 mm high were used for explant culture, each containing 8 ml of medium. Five explants were placed in each Petri dish and 10 Petri dishes were used per treatment (50 explants). Petri dishes were sealed with laboratory sealing film. Explants and calli were subcultured at 60-d-intervals and maintained in a climatic chamber at 25 °C, 16 $h \cdot d^{-1}$ light (photosynthetic photon flux density: 40 μmol m^{-2} s^{-1} cool-white fluorescent lamps).

Plant regeneration and *in vivo* acclimation: Explants and calli that differentiated somatic embryos were transferred to hormone-free NN medium supplemented with 88 mM sucrose (NN-) and incubated for 1-2 months to allow embryo proliferation and development. To allow germination of the embryos regenerated from anther and stigma-style explants, 10 embryos were placed on 8 ml of NN- medium in a plastic Petri dish (55 x 15 mm). Two hundred embryos per type of explant were used to calculate the percentage of embryo germination and conversion of embryos into plants. Embryos were considered as germinated when there was root extension and hypocotyl elongation. After germination, to allow the conversion of embryos into plants, two germinated somatic embryos were transferred to NN- medium (each 100 x 20 mm Petri dish containing 25 ml of medium). Embryos were considered converted into plants when the shoot apex developed and formed new expanded leaves. Plantlets were transferred to autoclaved Jiffy® peat pellets and maintained in a basal heating bench at 25 °C and at high relative humidity (95-98 %). To estimate the growth of regenerated plants the average number of nodes and plant length was scored. In this experiment 40 plants for each explant type were used. The mean number of nodes and plant length were calculated after two months of growth in the basal heating bench. Subsequently, the plants were pricked into pots containing sterile soil, transferred to the greenhouse and exposed to natural daylight conditions at 22/27 °C (night/day).

Results

The 4 different hormonal combinations (reported in literature and chosen to test the embryogenic potential of stigma-style explants, Tab. 1) promoted the formation of soft translucent creamy-white callus. In fact, about 1-2 weeks after culture initiation in all tested media we observed callus proliferation, while the hormone-free medium did not promote callus formation as expected. Callus became visible at the basal part of the style and consisted of cell clusters or single cells of variable size and shape.

About 3-4 months after culture initiation white somatic embryos were differentiated at the surface of callus regenerated from responsive explants (Fig. 1). Tab. 2 shows the embryogenic response of explants incubated on 4 different media scored after 6 months of incubation. Both explants regenerated somatic embryos only in the medium supplemented with 0.9 μM BA and 5 μM NOA. The attempts to induce somatic embryogenesis from anther and stigma-style explants cultured on the control (hormone-free medium) and the other media reported in literature were not successful (Tab. 2).

The second set of experiments was carried out to compare different levels of growth regulators which in the first set of experiments gave the best results. As in the first set of experiments, within the culture media used for the regeneration of somatic embryos in 4 species of the genus *Vitis*, only

Table 1

Hormonal combinations for regeneration of somatic embryos from different *Vitis* species reported in literature

Species	Explant type	Hormonal combination		GA_3	Reference
		Auxin compound and concentration	Cytokinin compound and concentration		
V. rotundifolia	Immature zygotic embryos	0.9 μM BA	5.0 μM NOA		GRAY 1992
V. rupestris	Leaf and petiole	4.4 μM BA	0.5 μM 2,4-D		MARTINELLI *et al.* 1993
V. vinifera	Stenospermocarpic ovules	1 μM KIN	10 μM IAA	1 μM GA_3	TSOLOVA and ATANASSOV 1994
V. thunbergii	Leaf discs	4 μM TDZ	20 μM NOA		HARST 1995

BA = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; GA_3 = gibberellic acid; IAA = 3-indoleacetic acid; KIN = kinetin; NOA = 2-naphthyloxyacetic acid; TDZ = Thiadiazuron.

Table 2

Embryogenic anthers (%) and embryogenic stigmas-styles (%) after incubation on 4 different media. The embryogenic response is expressed as mean percentage scored 6 months after culture initiation. Each treatment comprised 50 explants

Hormonal combination				
Auxin concentration	Cytokinin concentration	GA_3	Embryogenic anthers (%)	Embryogenic stigmas-styles (%)
0.9 µM BA	5.0 µM NOA		7	3
4.4 µM BA	0.5 µM 2,4-D		0	0
1 µM KIN	10 µM IAA	1 µM GA_3	0	0
4 µM TDZ	20 µM NOA		0	0
Control hormone free medium			0	0

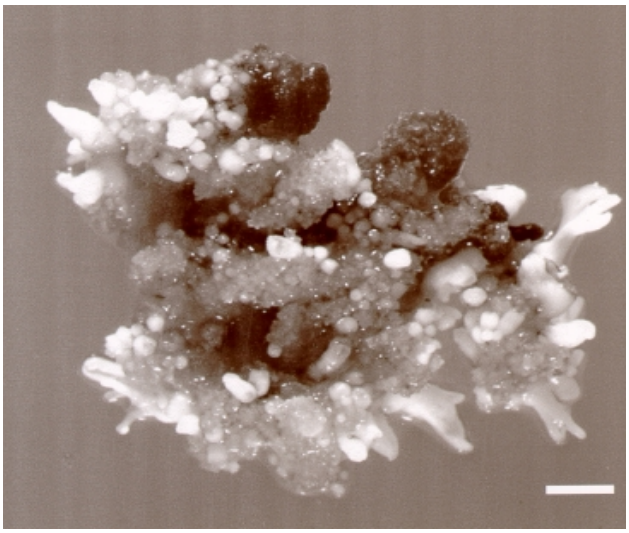

Fig. 1: Somatic embryos (small white protuberances) regenerated from stigma and style cultures. Bar = 2 mm.

the hormonal combination 0.9 µM BA and 5 µM NOA gave best results, 16 different combinations of these two growth regulators were used. Tab. 3 shows the embryogenic response of anther and stigma-style explants incubated on these media. The embryogenic response depended on explant type and growth regulator combination. Both explants regenerated somatic embryos, although they showed a different embryogenic aptitude. Stigma-style explants showed highest embryogenic potential as compared to anthers. The embryogenic response of explants was also affected by the hormonal composition of the medium. The higher percentage of stigma-style explants producing somatic embryos were 20 % on 9.0 µM BA + 5 µM NOA supplemented medium and 16 % on 9.0 µM BA + 10 µM NOA. The percentage of embryogenic anthers was 8 % on 4.5 µM BA + 5 µM NOA supplemented medium and 5 % on 4.5 µM BA + 10 µM NOA (Tab. 3). Addition of BA and NOA was necessary to induce regeneration of somatic embryogenesis, in fact it was never observed in explants incubated on hormone-free medium (Tab. 3).

A high percentage (about 60 %) of embryos showed a bipolar organisation, after incubation on growth regulator-free medium, they usually passed through globular, heart, torpedo and early cotyledon stages, and finally germinated embryos (Fig. 2). During embryo development we observed a change in colour from white to green.

Fig. 2: Somatic embryos after incubation on growth regulator-free medium passed through different stages of development. From left: globular, heart, torpedo, cotyledon and germinated embryos. Bar = 3 mm.

Table 3

Effects of the cytokinin BA and the auxin NOA on induction of somatic embryos from anther and stigma-style explants. For details: Tab. 2

	NOA (µM)	BA (µM)			
		0	0.5	4.5	9.0
Anthers	0	0	0	0	0
	1	0	0	4	0
	5	0	4	8	0
	10	0	0	5	0
Stigmas / styles	0	0	0	4	0
	1	0	0	0	8
	5	0	2	0	20
	10	0	0	12	16

The average number of primary somatic embryos produced directly from the callus derived from anther and stigma-style explants was low, *i.e.* 9 and 6, respectively. However, repetitive somatic embryogenesis - which comprises successive cycles of somatic embryogenesis induced from the hypocotyl cells of regenerated plants - allowed the rescue of many embryos. About 35 % of primary somatic embryos produced secondary somatic embryos. Secondary somatic

embryos were regenerated from the area surrounding the hypocotyl (Fig. 3). Embryogenic callus consisted of white proembryogenic masses (PEMs). When PEMs were maintained on induction medium the cytokinin and the auxin in the medium had an inhibitory effect on their development into somatic embryos. We observed that embryogenic callus can be subcultured for long time (> 2 years) maintaining its embryogenic potential.

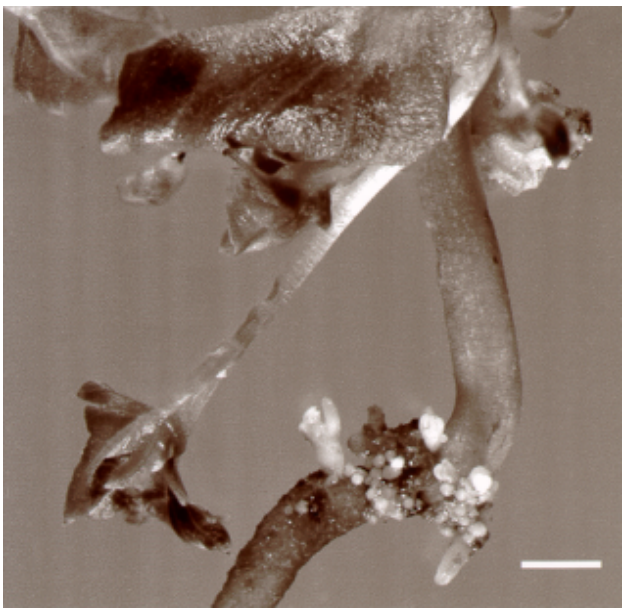

Fig. 3: Secondary somatic embryos regenerated from the area surrounding the hypocotyl. Bar = 2 mm.

Preglobular stage PEMs subcultured at low densities on hormone-free medium continue their development (globular, heart, torpedo and cotyledonary stage embryos). The percentage of somatic embryos derived from anther and stigma-style explants that germinated within one month after transfer to hormone-free medium was 52 and 46 %, respectively. The percentage of germinated embryos derived from anther and stigma-style explants converted to rooted plantlets within 3 months after embryo germination was 34 and 38 %, respectively.

Germinated somatic embryos incubated on hormone-free medium developed into plantlets that reached about 5-7 cm length within the first months of culture. Both explants developed to morphologically normal plants with well-expanded leaves. Plantlets that developed a high shoot and root system under *in vitro* conditions were chosen and pricked into autoclaved Jiffy® pellets. Rooted plantlets regenerated from anther and stigma-style explants showed a high survival rate; in fact, 82 and 87 % of regenerated plants survived the transfer from *in vitro* to *in vivo* conditions, respectively. After two month of growth in the basal heating bench the plant length was on average about 32 cm and the average number of nodes was 12.3; once transferred to the greenhouse they developed new leaves and continued to grow (Fig. 4).

To test if the regeneration of somatic embryos from stigma and style explant was a reliable regeneration protocol, stigma and style explants were collected the third year of trial, and they were incubated only on 9 µM BA + 5 µM NOA or 9 µM BA + 10 µM NOA supplemented media; the percentages of embryogenic explants were 27 and 19, respectively.

Fig. 4: Plant regenerated from stigma and style culture pricked into pot and growing in the greenhouse. Bar = 10 cm.

Discussion

The present study indicates a high embryogenic potential of stigma and style tissues of cv. Sugraone. Although regeneration of somatic embryos from anther culture in grapevine is generally considered efficient and is the most commonly used method (BOUQUET and TORREGROSA 2003), stigma and style explants showed highest embryogenic potential as compared to anthers. In both explants the tested growth regulators were required for induction of somatic embryogenesis. However, the cytokinin and auxin levels required for the induction of somatic embryogenesis, depended on the explant type. Under our experimental conditions, anthers required lower levels of cytokinin (4.5 µM) in the culture medium as compared with stigmas and styles (9 µM). The highest embryogenic response was observed in stigma and style explants cultured on NN medium supplemented with 5 µM NOA and 9 µM BA (20 and 27 % of responsive explants).

Another part of the flower, the ovary, has already been reported as a valuable explant for induction of somatic embryogenesis in different species of *Vitis* (GRAY and MORTENSEN 1987, NAKANO *et al.* 1997) and in some cases the embryo-

genic potential of this explant was higher as compared to anther explants (MARTINELLI *et al.* 2001a, 2003).

Since embryogenic calli obtained from stigma and style culture may have been derived solely from cells of somatic origin, the regenerated embryos have a genetic constitution that is identical to that of the plant source, excluding somaclonal variation. Usually, anther cultures can produce embryos that arise from diploid cells of the connective tissue and are therefore of somatic origin with a genome identical to that of the mother plant (FAURE *et al.* 1996, SALUNKHE *et al.* 1999). However, it has been reported that the embryos regenerated from anther culture may arise from the haploid cells of gametic origin (BOUQUET and TORREGROSA 2003).

Secondary embryogenesis from primary somatic embryos is a crucial aspect for a long time preservation of embryogenic cultures (VILAPLANA and MULLINS 1989, MARTINELLI *et al.* 2001 b). Under our experimental conditions a high percentage (35 %) of primary somatic embryos regenerated secondary somatic embryos. Secondary embryogenesis has several advantages when compared to primary somatic embryogenesis, such as a high multiplication rate, independence of explant availability (*i.e.* flowers or immature leaves are available only for a short period), moreover embryogenic cell lines can be maintained for several years (RAEMAKERS *et al.* 1995).

We have observed that the callus formed from stigma and style culture is either embryogenic or not. It is usually easy to distinguish between non-embryogenic and embryogenic callus on the base of its colour and morphology. Embryogenic callus consists of white PEMs.

In the present work, phenotypically normal cv. Sugraone plants have been regenerated. However, the embryogenic potential of stigma-style explants of other genotypes should be tested. Moreover, genetic identity of regenerated plants should also be confirmed in the field and at molecular level by molecular markers, to evaluate the somaclonal variation of plants regenerated from stigma and style explants.

Acknowledgements

This work was supported by the "Conservazione in vivo ed in vitro di varietà minori di fruttiferi in via di estinzione, tipici degli ambienti mediterranei", project of the Regione Siciliana, Assessorato Agricoltura e Foreste.

References

BOUQUET, A.; TORREGROSA, L.; 2003: Micropropagation of the grapevine (*Vitis* spp.). In: S. M. JAIN, K. ISHII (Eds): Micropropagation of Woody Trees and Fruits **75**, 319-352. Kluwer Academic Publishers, Dordrecht, The Netherlands.

DAS, D. K.; REDDY, M. K.; UPADHYAYA, K. C.; SOPORY, S. K.; 2002: An efficient leaf-disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). Plant Cell Rep. **20**, 999-1005.

FAURE, O.; AARROUF, J.; NOUGAREDE, A.; 1996: Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): Proembryogenesis. Ann. Bot. **78**, 23-28.

GOUSSARD, P. G.; WIID, J.; KASDORF, G. G. F.; 1991: The effect of *in vitro* somatic embryogenesis in eliminating fanleaf virus and leafroll associated viruses from grapevines. S. Afr. J. Enol. Vitic. **12**, 77-81.

GOUSSARD, P. G.; WIID, J.; 1992: The elimination of fanleaf virus from grapevines using *in vitro* somatic embryogenesis combined with heat therapy. S. Afr. J. Enol. Vitic. **13**, 81-83.

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

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

HARST, M.; 1995: Development of a regeneration protocol for high frequency somatic embryogenesis from explant of grapevines (*Vitis* spp.). Vitis **34**, 27-29.

JAYASANKAR, S.; LI, Z.; GRAY, D. J.; 2000: *In-vitro* selection of *Vitis vinifera* 'Chardonnay' with *Elsinoe ampelina* culture filtrate is accompanied by fungal resistance and enhanced secretion of chitinase. Planta **211**, 200-208.

MARTINELLI, L.; BRAGAGNA, P.; POLETTI, V.; SCIENZA, A.; 1993: Somatic embryogenesis from leaf- and petiole-derived callus of *Vitis rupestris*. Plant Cell Rep. **12**, 207-210.

MARTINELLI, L.; CANDIOLI, E.; COSTA, D.; POLETTI, V.; RASCIO, N.; 2001 b: Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history. Plant Cell Rep. **20**, 279-284.

MARTINELLI, L.; GRIBAUDO, I.; 2001: Somatic embryogenesis in grapevine (*Vitis* spp.) In: K. ROUBELAKIS-ANGELAKIS (Ed.): Molecular Biology and Biotechnology of Grapevine, 327-352. Kluwer Academic Publishers, Dordrecht, The Netherlands.

MARTINELLI, L.; GRIBAUDO, I.; BERTOLDI, D.; CANDIOLI, E.; POLETTI, V.; 2001 a: High efficiency somatic embryogenesis and plant germination in grapevine cultivars Chardonnay and Brachetto a grappolo lungo. Vitis **40**, 111-115.

MARTINELLI, L.; GRIBAUDO, I.; SEMENZATO, M.; POLETTI, V.; 2003: Ovary as valuable explant for somatic embryogenesis induction in grapes (*Vitis* spp.). Acta Hortic. **603**, 499-504.

MAURO, M. C.; NEF, C.; FALLOT, J.; 1986: Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon. Plant Cell Rep. **5**, 377-380.

MULLINS, M. G.; SRINIVASAN, C.; 1976: Somatic embryos and plantlets from an ancient clone of the grapevine (cv. Cabernet-Sauvignon) by apomixis *in vitro*. J. Exp. Bot. **27**, 1022-1030.

NAKAJIMA, I.; MATSUTA, N.; 2003: Somatic embryogenesis from filaments of *Vitis vinifera* L. and *Vitis labruscana* Bailey. Vitis, **42**, 53-54.

NAKANO, M.; SAKAKIBARA, T.; WATANABE, Y.; MII, M.; 1997: Establishment of embryogenesis cultures in several cultivars of *Vitis vinifera* and *V.* x *labruscana*. Vitis **36**, 141-145.

NAKANO, M.; WATANABE, Y.; HOSHINO, Y.; 2000: Histological examination of callogenesis and adventitious embryogenesis in immature ovary culture of grapevine (*Vitis vinifera* L.). J. Hort. Sci. Biotech. **75**, 154-160.

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

RAEMAKERS, C. J. J. M.; JACOBSEN, E.; VISSER, R. G. F.; 1995: Secondary somatic embryogenesis and applications in plant breeding. Euphytica **81**, 93-107.

SALUNKHE, C. K.; RAO, P. S.; MHATRE, M.; 1997: Induction of somatic embryogenesis and plantlets in tendrils of *Vitis vinifera* L. Plant Cell Rep. **17**, 65-67.

SALUNKHE, C. K.; RAO, P. S.; MHATRE, M.; 1999: Plantlet regeneration via somatic embryogenesis in anther callus of *Vitis latifolia* L. Plant Cell Rep. **18**, 670-673.

STAMP, J. A.; MEREDITH, C. P.; 1988 a: Proliferative somatic embryogenesis from zygotic embryos of grapevines. J. Am. Soc. Hort. Sci. **113**, 941-945.

STAMP, J. A.; MEREDITH, C. P.; 1988 b: Somatic embryogenesis from leaves and anthers of grapevine. Scientia Hort. **35**, 235-250.

TSOLOVA, V.; ATANASSOV, A.; 1994: Induction of polyembryony and secondary embryogenesis in culture for embryo rescue of stenospermocarpic genotypes of *Vitis vinifera* L. Vitis **33**, 55-56.

VILAPLANA, M.; MULLINS, M. G.; 1989: Regeneration of grapevine (*Vitis* spp.) *in vitro*: Formation of adventitious buds on hypocotyls and cotyledons of somatic embryos. J. Plant Physiol. **134**, 413-419.

Received February 13, 2004