Vitis **47** (3), 169–174 (2008)

High frequency occurrence of single cotyledonary embryo morphotype and repetitive somatic embryogenesis in 'Thompson Seedless' crossed with seven grapevine male parents

P. V. Bharathy and D. C. Agrawal

Plant Tissue Culture Division, National Chemical Laboratory, Pune, India

Summary

Direct somatic embryogenesis was observed in zygotic embryos rescued from intra- and inter-specific crosses between 'Thompson Seedless' and seven male parents of grapevine when the embryos were cultured on Woody Plant Medium (WPM) supplemented with benzyladenine (1 µM). Repetitive somatic embryogenesis occurred on the same medium, which also supported a high percentage of embryo maturation, germination and plantlet development. The cultures retained embryogenic potential for more than two years.

We observed a high frequency occurrence of monocotyledonous embryo morphotype and other morphological variations in somatic embryos of all the crosses. The percentage of embryos having mono-, di-, tri-, multiple and abnormal cotyledons varied among the crosses. The overall percentage of monocot embryos was 35.50 %, as against 38.64 % of dicot embryos, while the germination rates for mono- and dicot- embryos were 24.44 % and 24.15 %, respectively. Shoot development was poor in tri- and multiple-cotyledonary embryos, while there was no shoot formation in abnormal embryos. We assume that the relatively high occurrence of single cotyledonary morphotype may be due to the repetitive exposure of embryogenic tissues to a medium containing benzyladenine.

K e y w o r d s : cotyledon, grapevine, somatic embryo, *Vitis*.

A b b r e v i a t i o n s : BA: Benzyladenine, WPM: Woody Plant Medium, TS: 'Thompson Seedless'.

Introduction

Somatic embryogenesis has served as a useful regeneration method for genetic transformation and an excellent *in vitro* model system for investigating regulatory mechanisms responsible for gene expression related to embryo development. Since Mullins and Srinivasan (1976) reported somatic embryogenesis in the highly recalcitrant grape for the first time, several studies have revealed the successful initiation and establishment of embryogenic cultures in numerous grapevine cultivars using various explants, different culture conditions, basal media and hormonal composition. However, the technique is still not routine and often problems like low induction efficiency, low morphogenetic competence and poor germination and plant conversion rates, especially in long-term cultures have been encountered (Martinelli and Gribaudo 2001).

Improper development of embryonic cotyledons is a good tool to study abnormalities encountered during somatic embryogenesis. Variations in embryo morphology, particularly number of cotyledons and genetic studies have earlier been reported only in a few species like *Arabidopsis* (Jürgens *et al.* 1991, Aida *et al.* 1997, Aida *et al.* 2002) and larch (Harrison and von Aderkas 2004). In embryos of zygotic origin, embryo size, genetic heritability, phenotypic variation and gene expression seem to control cotyledon number in a particular taxon (Aida *et al.* 2002). Somatic embryos are more susceptible to variation in cotyledon number than their zygotic counterparts. This may be due to a number of factors like culture conditions, growth regulators and basal medium. Variability in cotyledon number in somatic embryos due to application of plant growth regulators like benzyladenine (von Aderkas 2002) and anti-auxin 2,3,5-triiodobenzoic acid (Choi *et al.* 1997) has been reported.

During our study to induce downy mildew resistance in 'Thompson Seedless' using embryo rescue technique, we observed *de novo* somatic embryogenesis from zygotic embryos of crosses between TS and seven male parents on Woody Plant Medium supplemented with 1 µM BA. Repetitive somatic embryogenesis or induction of new somatic embryos from pre-existing embryos occurred when these embryos were transferred to the fresh medium. We report a relatively simple procedure for long-term maintenance of the embryogenic competence of the cultures and conversion of embryos into plantlets. During experiments to improve the embryogenic response, we observed a high frequency occurrence of embryos having a single cotyledon in all the crosses, along with the occurrence of embryos with variable cotyledon number. The single cotyledonary embryo was generally larger in size than the conventional dicotyledonary embryos and had a good rate of plant regeneration. The present study was carried out to characterize the different types of embryos observed during our experiments, with more stress on the monocotyledonous embryo development compared to the normal dicotyledonous embryos.

Correspondence to: Dr. D. C. Agrawal, Plant Tissue Culture Division, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune – 411 008, India. Fax: +91-20-2590-2645. E-mail: dc.agrawal@ncl.res.in

Materials and Methods

Plant material: Twenty one vines of 'Thompson Seedless' (TS) susceptible to downy mildew (*Plasmopara viticola* Berl. and de Toni), selected as female parent were crossed with seven male parental lines (seeded) of grapevine showing field tolerance to downy mildew. The seven male parents selected were as follows: 'Concord', 'Catawba' (belonging to *Vitis labrusca*), 'Frühroter Veltliner' (*Vitis vinifera*), 'Seyve Villard - S.V. 18402' (*Vitis* spp.), *Vitis tilifolia, Vitis candicans* and 'St. George' (*Vitis rupestris* du Lot). Emasculation in 'Thompson Seedless' was carried out followed by immediate bagging of emasculated panicles. Next morning, the flowers were hand pollinated with the designated male pollen, collected and stored earlier, using a separate hair brush for each cross. Berries from crosses were collected 40 d after pollination. These were washed with liquid soap for 10 min, then disinfected with mercuric chloride (0.1 % w/v) solution for 10 min and finally rinsed three times with sterile distilled water.

Ovule and embryo culture: Ovules from the berries were aseptically excised and cultured in petri dishes (55 mm ø) containing Emershad and Ramming medium (ER) (Emershad and Ramming 1984), with sucrose (6 %), activated charcoal (0.3 %) and agar (0.65 %). All chemicals used in the study were purchased from Qualigens, Glaxo India Limited, unless otherwise specified. The pH of the medium was adjusted to 5.8 before autoclaving. Each petri dish (55 mm ø) contained 15-18 ovules. The dishes were kept under 24 h photoperiod with diffused light intensity of 6.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 25 $\pm$ 2 °C for 10 weeks to allow the development of embryos within the ovule.

After 10 weeks of incubation, the ovules were dissected aseptically under microscope and embryos were excised from the micropylar end of the ovule. The embryos were transferred to petri dishes (55 mm ø) containing Woody Plant Medium (WPM) (Lloyd and McCown 1981), supplemented with BA (1 μM) (Sigma, St. Louis, Mo.), sucrose (1.5 %), activated charcoal (0.3 %) and agar (0.65 %). pH of the medium was adjusted to 5.8. These were incubated at 25 $\pm$ 2 °C under permanent fluorescent light (12.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for germination and plant development.

Induction of repetitive somatic embryogenesis and long-term culture maintenance: Somatic embryos that arose from zygotic embryos were plated in petri dishes (55 mm ø) containing WPM with same composition and culture conditions as for embryo culture. Subculture of secondary embryos to fresh plates of WPM was carried out every 8 weeks.

Morphology of embryos: Clumps of embryos in advanced stage of development were plated in petri dishes (55 mm ø) containing WPM supplemented with 1 µM BA, 1.5 % sucrose, 0.3 % activated charcoal, 0.65 % agar and pH of 5.8. Each cross was replicated at least five times, depending on the availability of embryos. The cultures were incubated under 24 h photoperiod (12.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25 $\pm$ 2 °C. Observations like number of cotyledons per embryo of each cross combination and other abnormal features of embryos were recorded after 1 week under zoom stereomicroscope (Leica, Switzerland). Means and standard deviation were calculated for the data for statistical analysis.

Embryo germination and plantlet development: To assess germination and plant development, embryos were picked randomly and plated in petri dishes (85 mm ø) containing WPM with same composition as above. Ten embryos of each cross were plated per petri dish with minimum 3 replicates. The petri dishes were kept under 24 h photoperiod (12.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25 $\pm$ 2 °C. In order to develop into plants, germinating somatic embryos showing shoot growth were transferred to test tubes containing WPM supplemented with BA (1 μM) and kept under similar light and temperature conditions. After 4 weeks, the plantlets were transferred to plastic cups containing a mixture of soil + sand (1:1). For hardening, procedure described by Bharathy *et al.* (2003) was followed. Number of plants established was recorded. For the statistical analysis, averages and standard errors of means of the parameters studied were calculated.

Histology: For histological studies, embryos were fixed in formalin: acetic acid: ethanol (5:5:90 v/v) for 48 h. Tissues were dehydrated stepwise by passing through tertiary-butanol series, followed by embedding in paraffin wax (58-60 °C). Embedded tissues were cut into 10 mm thick sections, using a rotary microtome. The sections were de-waxed and stained with haematoxylin-eosin, mounted with DPX-4 189-[2-chloro-N-(4-methoxy-6 methyl-1,3-5-triazin-2yl)-aminocarbonyl] benzene sulfonamide and were observed under microscope.

Results and Discussion

Induction of repetitive somatic embryogenesis: Repetitive direct somatic embryogenesis was observed in somatic embryos of all the seven crosses and open pollinated embryos (Fig. 1 A) when the embryos were transferred to fresh Woody Plant Medium. The choice of the basal medium was critical for formation of embryos as well as for maintenance of embryogenic potential, as other basal media showed little or no response (data not presented). WPM supported induction of embryogenesis as well as plant development. Adventitious embryos developed on the hypocotyls at the root-shoot zone (Fig. 1 B), cotyledon and sometimes from the entire embryo. Somatic embryos induced mostly on abnormal embryos which were not capable of developing into plants. These embryos formed *de novo*, mostly without callus formation and could be separated easily. Embryos developed asynchronously (Fig. 1 C). Somatic embryos were larger and looked more vivid than their zygotic counterparts. Depending on the physiological state, the embryos either developed into plants or induced a new crop of somatic embryos, but sometimes even normally developing embryos gave rise to secondary embryos at the root-shoot zone. The embryos germinated easily on WPM (Fig. 1 D) and often precocious germination of incompletely developed

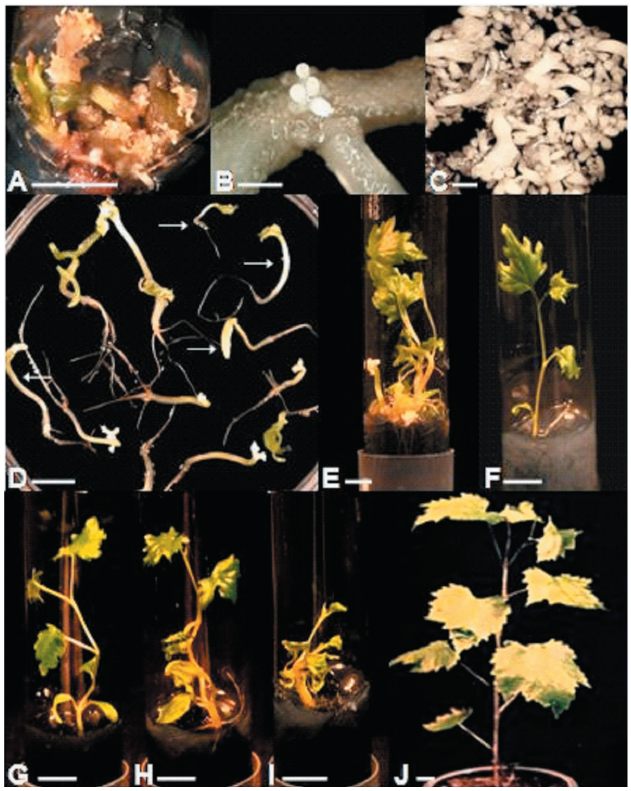

Fig. 1: Repetitive somatic embryogenesis in 'Thompson Seedless'. **A**: In culture. **B**: Somatic embryos arising from hypocotyls (bar 1 mm). **C**: Asynchronous somatic embryos (bar 1mm). **D**: Germinating somatic embryos, arrows point to the monocot morphotypes. **E**: Plantlets in tube. **F-I**: Plantlets from **F**: One, **G**: Two, **H**: Three, **I**: Multiple cotyledonary embryos. **J**: Hardened plant. (*bar* corresponds to 1 cm for remaining photos)

embryos was observed. Germinating somatic embryos were transferred to test tubes containing WPM (Fig. 1 E) for plantlet development.

The successful maintenance of embryogenic competence of cultures along with high efficiency of conversion into plantlets is of utmost importance in plant tissue culture. In our study, WPM supplemented with 1 µM BA was highly efficient for achieving the quadruple objectives of embryogenic culture maintenance, embryo maturation, embryo germination and plant development. Embryo maturation and germination could be carried out successfully on the same medium, probably due to the addition of activated charcoal, which has been known to aid in somatic embryo induction (Lopez-Perez *et al*. 2005), embryo maturation (Motoike *et al.* 2001) and counteract the deleterious effects of phenolic compounds secreted by the plant tissues (Zhu *et al.* 1997). Emershad and Ramming (1994) working with *V. vinifera* have earlier reported the occurrence of somatic embryogenesis in immature zygotic embryos *in-ovulo* when cultured on liquid Emershad and Ramming medium, while induction of secondary somatic embryogenesis was observed on the same medium with 1 µM BA and plant development occurred on WPM supplemented with 1 µM BA.

Morphology and histology of somatic embryos: A high frequency occurrence of monocotyledonary morphotype was observed in all cross combinations and was highly significant among the cross combinations (Tab. 1). In open pollinated TS, the percentage of mono-, di-, tri- and multiple cotyledonary embryos (Fig. 2 A-D, inset-embryos in culture) was 37.04 %, 39.31 %, 1.93 % and 8.33 % respectively. The percentage of abnormal embryos was 13.39 %. Among cross combinations, TS x 'Concord' and TS x 'Frühroter Veltliner' showed a high percentage of single cotyledonary embryos (36.91 %) as against 31.54 % normal dicotyledonary embryos. In contrast, in TS x 'Catawba', TS x 'SV 18402' and TS x *V. candicans*, the percentage of dicotyledonous embryos was more (43.60 %, 36.80 % and 36.75 %, respectively) than monocotyledonous embryos (32.80 %, 33.60 %, 23.80 % respectively). The percentage of tricotyledonary embryos ranged from 2.67 % to 4.40 %, while for multiple cotyledonary embryos, it ranged from 2.80 to 9.40 %. The overall percentage of monocot embryos was 35.50 %, as against 38.64 % of dicot embryos. Histology of the embryos revealed the complete absence of a cotyledon in the monocotyledonous embryo (Fig. 3 A). In dicot embryos, the vascular strands ended in the cotyledons (Fig. 3 B, E) while in the monocot morphotype, one strand ended in the shoot meristem (Fig. 3 D). Staining in the meristematic region of monocot morphotype was more dense and deep than that of the dicot embryo, revealing more cell layers of meristematic tissue.

Table 1

Variation in number of cotyledons in somatic embryos of crosses of 'Thompson Seedless' and 5 male parents in culture

No.	Cross 'Thompson Seedless' X	Number of somatic embryos used	Percentage of somatic embryos having different number of cotyledons				
			One (Mono)	Two (Di)	Three (Tri)	Multiple	Abnormal
1	Open-pollinated (*V. vinifera*)	1501	37.04	39.31	1.93	8.33	13.39
2	'SV 18402' (*Vitis* sp.)	250	33.60	36.80	4.00	2.80	22.80
3	*V. candicans*	117	23.08	36.75	3.42	9.40	27.35
4	'Concord' (*V. labrusca*)	149	36.91	31.54	3.36	4.03	24.16
5	'Catawba' (*V. labrusca*)	250	32.80	43.60	4.40	6.80	12.40
6	'Frühroter Veltliner' (*V. vinifera*)	150	36.00	35.33	2.67	6.67	19.33
	Mean ± S.D.		35.50 ± 5.28	38.64 ± 4.03	2.61 ± 0.89	7.28 ± 2.51	15.97 ± 6.02

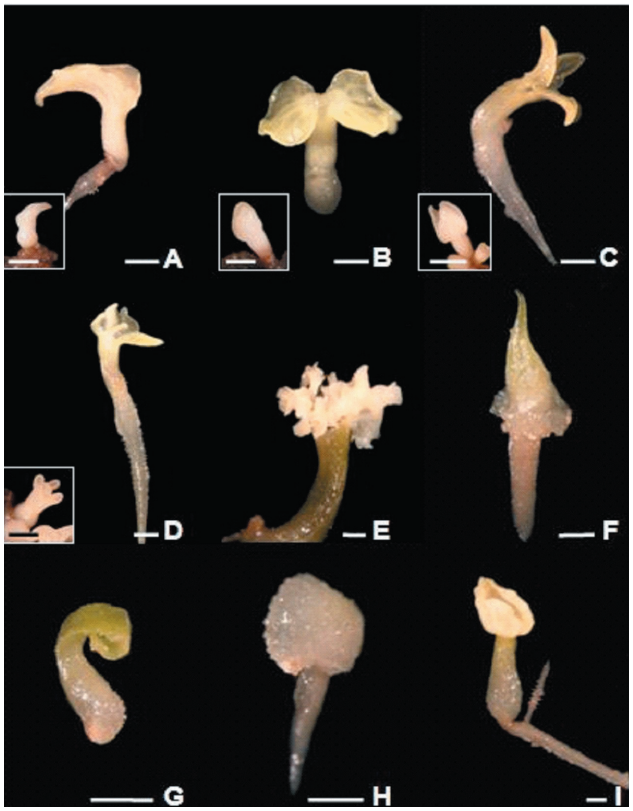

Fig. 2: Embryos with **A**: One, **B**: Two, **C**: Three, **D**: Multiple cotyledons. **E-I**: Abnormal embryos. **E**: Cabbage-like. **F**: Without cotyledon. **G**: Without root. **H**: Totally lacking distal embryo parts. **I**: Cup-shaped (bar 2 mm). Insets **A-D**: Embryos in culture (bar 1 mm).

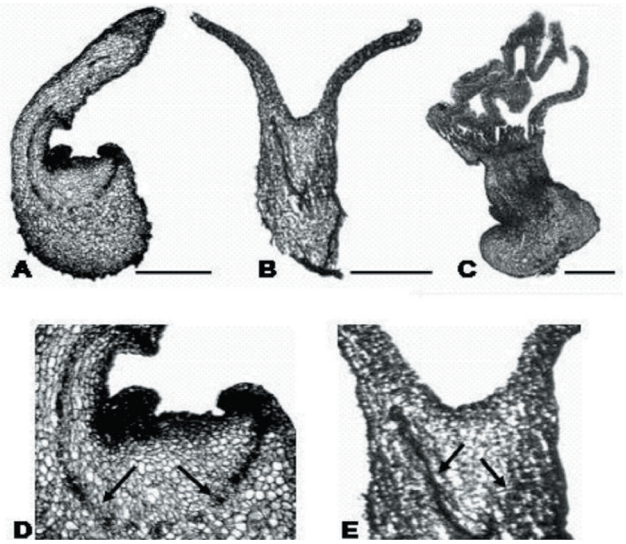

Fig. 3: Histology of embryos with - **A**: Single (bar 500 µm). **B**: Two (bar 500 µm). **C**: Multiple (bar 1 mm) cotyledons. **D**: Monocot embryo - the arrows point to the vascular strands ending in one of the two buds (magnified 200X). **E**: Dicot embryo - The arrows point to vascular strands (magnified 200X).

The phenomenon of unusually high occurrence of monocotyledonary morphotype obtained in the present study could be a result of repetitive subculture on a medium containing BA. These observations are in agreement with that of von Aderkas (2002), who reported that BA caused the greatest reduction in average cotyledon number per embryo when applied exogenously in hybrid larch and at concentrations above 4.4 µM, BA reduced the percentage of embryos able to initiate cotyledons. In another report by Harrison and von Aderkas (2004), it was revealed that the number of cotyledons in hybrid larch was linearly related to the diameter of the apical surface of the embryo (a circular disc) where cotyledon primordia first appeared. They reported that BA reduced the number of embryos having a diameter above 300 µm, and thereby the average number of cotyledons. In our study, the combined effect of endogenous hormone levels, exogenous addition of BA to the medium, long term culture on medium containing BA and an alteration in the defined embryo development sequence may have resulted in a high frequency of the single cotyledonary morphotype.

Abnormal embryos were distinguished from the other morphotypes on the basis of their morphology and the inability to develop into plants. The percentage of abnormal embryos was rather high in TS x *V. candicans* (27.35 %) and TS x 'Concord' (24.16 %), while in other cross combinations, it ranged from 12.40 to 22.80 %. Abnormal embryos were either cabbage-like (Fig. 2 E), without cotyledons (Fig. 2 F), without root (Fig. 2 G) or complete absence of the shoot meristem (Fig. 2 H). The complete absence of root or shoot meristem in embryos maybe due to delayed establishment of polarity (Halperin 1964). The cellular organization in cabbage-shaped embryo (Fig. 3 C) showed dense cytoplasm with least vacuolated area with no clear distinction of the vascular strands and the shoot meristematic region. Many times, embryos with fused, cup-shaped cotyledons (Fig. 2 I) were also observed. These embryos remained white and did not develop into plantlets. West and Harada (1993) reported that polar auxin transport was specifically necessary at the globular stage of embryos in order to produce two cotyledonary lobes and embryos defective in auxin transport developed fused, cup-shaped cotyledons. Lopez-Perez *et al*. (2006) reported the occurrence of various embryo morphotypes on medium containing IAA + GA_3.

Embryo germination and plantlet development: The embryo germination and plant conversion rates varied among the open-pollinated and six cross combinations (Tab. 2). Overall, the germination rates (Tab. 2) were almost similar for monocot (Fig. 1 F) and dicot (Fig. 1 G) embryos (24.44 % and 24.15 %, respectively), though monocot embryos gave rise to shoot earlier than the dicot morphotype (data not presented). The absence of second cotyledon did not hamper the normal development of the monocot morphotype and monocot embryos had a longer hypocotyl and enlarged cotyledons (Fig. 1 D) than dicot embryos. For both the types, root system was a tap root. For monocot (41.94 %) and dicot (37.78 %) embryos from the open-pollinated TS, a germination rate of 36.11 % and 31.94 % were obtained. In contrast to these, in the cross TS x *V. candicans*, germination rates of both morphotypes were low, 1.67 % (from 25 % monocots) and 6.67 % (from 38.33 % dicots). Plants developed from both the morphotypes (monocot and dicot) showed no differences in morphological characters and were similar to as shown in Fig. 1 J. The higher germination of the monocot

Table 2

Germination percentage of different embryo morphotypes in crosses of 'Thompson Seedless' and 6 male parents

No.	Cross 'Thompson Seedless' x	Number of somatic embryos used	Germination percentage of somatic embryos with different cotyledon numbers*				
			One	Two	Three	Multiple	Abnormal
1	Open pollinated (*V. vinifera*)	360	41.42 ± 5.03	36.95 ± 2.66	4.00 ± 1.04	5.63 ± 0.82	16.54 ± 3.72
2	'St.George' (*V. rupestris du Lot*)	45	36.11 ± 2.63	31.94 ± 3.88	0.56 ± 0.17	2.78 ± 1.83	0.00 ± 0.00
			37.78 ± 6.29	40.00 ± 3.44	8.89 ± 3.47	6.67 ± 7.07	6.67 ± 3.54
3	*V. candicans*	60	22.22 ± 9.46	17.78 ± 4.41	2.22 ± 2.89	4.44 ± 4.71	0.00 ± 0.00
			25.00 ± 3.85	38.33 ± 2.22	1.67 ± 2.72	13.33 ± 5.44	21.67 ± 5.88
4	*V. tilifolia*	30	1.67 ± 1.67	6.67 ± 6.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
			36.67 ± 3.33	30.00 ± 3.33	0.00 ± 0.00	6.67 ± 6.67	26.67 ± 6.67
5	'Concord' (*V. labrusca*)	30	6.67 ± 0.00	10.00 ± 3.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
			53.33 ± 6.67	26.67 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	20.00 ± 6.67
6	'Catawba' (*V. labrusca*)	80	10.00 ± 10.00	6.67 ± 6.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
			26.25 ± 5.54	36.25 ± 6.25	8.75 ± 3.82	6.25 ± 1.44	22.50 ± 11.46
7	'Frühroter Veltliner' (*V. vinifera*)	70	8.75 ± 5.54	18.75 ± 6.57	3.75 ± 4.33	1.25 ± 1.44	0.00 ± 0.00
			34.29 ± 3.99	37.14 ± 7.07	7.14 ± 1.98	10.00 ± 1.97	11.43 ± 4.41
	Mean		17.14 ± 4.58	22.86 ± 7.56	2.86 ± 1.72	1.43 ± 1.25	0.00 ± 0.00
			37.78 ± 2.75	36.89 ± 9.33	4.15 ± 7.07	5.78 ± 7.09	15.41 ± 7.03
			24.44 ± 4.65	24.15 ±2.91	1.19 ± 2.48	2.07 ± 0.16	0.00 ± 0.00

* The top row under each category of cotyledons represents percentage of embryo morphotype observed (out of number of somatic embryos used). While, bottom row represents percentage germination and plant development (out of number of somatic embryos used).

embryos may be the result of better supply of nutrients to the shoot meristem due to the termination of one of the vascular strands into this zone. Results obtained in the present study are in agreement with Jayashankar *et al.* (2002) who obtained higher germination in single cotyledonary embryos (70 %) than dicot embryos (22 %) in first 4 weeks but after 6 weeks of culture, there was not much difference.

Shoot development in tri- and multiple-cotyledonary embryos was poor (Fig. 1 H, I) and in some cases growth of embryo itself was inhibited. The poor shoot formation in these embryo morphotypes may be due to a misallocation of apical cells into cotyledon forming fields as reported by Vernon *et al*. (2001). In abnormal embryos, no shoot formation occurred in the crosses. The inhibition of shoot development in abnormal embryos may be due to the improper development of vascular strands and shoot meristematic regions as revealed in histological studies of the cabbage type morphotype. Thus, though the cotyledons and shoot primordia are formed during the sequential development pathway in embryos, an interplay of different factors may lead to abnormalities in embryo development, more so in somatic embryos.

Thus, the present communication has described direct somatic embryogenesis from zygotic embryos of 'Thompson Seedless' crossed with seven seeded parents of grapevine including two inter-specific crosses *V. vinifera x V. candicans and V. vinifera* x *V. tilifolia* for the first time. Woody Plant Medium (WPM) supplemented with benzyladenine (1 µM) was found suitable for obtaining a repetitive proliferation of embryos and maintaining the embryogenic potential of the cultures for more than 2 years. Embryo maturation, germination and plant conversion occurred on the same medium with a high percentage. The study characterizes the variation in cotyledon number and abnormal embryo morphotypes in somatic embryos that arose during *in vitro* culture over a long culture period on a medium containing BA.

Acknowledgements

The financial assistance in the form of a research grant by the National Agriculture Technology Programme (NATP), ICAR, New Delhi; a Senior Research Fellowship to Mrs. Bharathy by Council of Scientific & Industrial Research (CSIR), Govt. of India and the help in form of base plant material by National Research Centre for Grapes (NRCG), and Agharkar Research Institute (ARI), Pune are gratefully acknowledged.

References

Aderkas, P. von; 2002: *In vitro* phenotypic variation in larch cotyledon number. Int. J. Plant Sci. **163**, 301-307.

Aida, M.; Ishida, T.; Fukaki, H.; Fujisawa, H.; Tasaka, M.; 1997: Genes involved in organ separation in *Arabidopsis*: an analysis of cup-shaped cotyledon mutant. Plant Cell **9**, 841-857.

Aida, M.; Vernoux, T.; Furutani, M.; Traas, J.; Tasaka, M.; 2002: Roles of *PIN-FORMED1* and *MONOPTEROS* in pattern formation of the apical region of the *Arabidopsis* embryo. Development **129**, 3965-3974.

Bharathy, P. V.; Karibasappa, G. S.; Biradar, A. B.; Kulkarni, D. D.; Solanke, A.U.; Patil, S.G.; Agrawal, D.C.; 2003. Influence of pre-blossom sprays of benzyladenine on *in vitro* recovery of hybrid embryos from crosses of Thompson Seedless and eight seeded varieties of grape (*Vitis* spp.). Vitis **42**, 199-202.

Choi, Y. E.; Kim, H. S.; Soh, W. Y.; Yang, D. C.; 1997: Developmental and structural aspects of somatic embryos formed on medium containing 2,3,5-triiodobenzoic acid. Plant Cell Reports **16**, 738-744.

Emershad, R. L.; Ramming, D. W.; 1984. *In ovulo* embryo culture of *Vitis vinifera* L. cv. Thompson Seedless. Am. J. Bot. **71**, 837-877.

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

Halperin, W.; 1964: Alternative morphogenetic events in cell suspensions. Am. J. Bot. **53**, 443-453.

Harrison, L. G.; von Aderkas, P.; 2004: Spatially quantitative control of the number of cotyledons in a clonal population of somatic embryos of hybrid larch *Larix* x *leptoeuropaea*. Ann. Bot. **93**, 423-434.

Jayasankar, S.; Bondada, B. R.; Li, Z.; Gray, D. J.; 2002: A unique morphotype of grapevine somatic embryos exhibits accelerated germination and early plant development. Plant Cell Rep. **20**, 907-911.

Jürgens, G.; Mayer, U.; Ruiz, R.; Berleth, T.; Misera, S.; 1991: Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development **91**, 27-38.

Llyod, G.; McCown, B.; 1981: Commercially feasible micropropagation of mountain laurel *Kalmia latifolia*, by use of shoot tip culture. Proc. Int. Plant Propagation Soc. **30**, 21-427.

Lopez-Perez, A. J.; Carreno, J.; Dabauza, M.; 2006: Somatic embryo germination and plant regeneration of three grapevine cvs: Effect of IAA, GA(3) and embryo morphology. Vitis **45**, 141-143.

Lopez-Perez, A.J.; Carreno, J.; Martinez-Cutillas, A.; Dabauza, M.; 2005: High embryogenic ability and plant regeneration of table grapevine cultivars (*Vitis vinifera* L.) induced by activated charcoal. Vitis **44**, 79-85.

Martinelli, L.; Gribaudo, I.; 2001: Somatic embryogenesis in grapevine. In: K. A. Roubelakis-Angelakis (Ed.): Molecular biology and biotechnology of the grapevine, 327-351. Kluwer Academic Publishers, the Netherlands.

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

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

Vernon, D. M.; Hannon, M. J.; Le, M.; Forsthoefel, N. R.; 2001: An expanded role for the *TWN*1 gene in embryogenesis: Defects in cotyledon pattern and morphology in the *twn*1 mutant of *Arabidopsis* (Brassicaceae). Am. J. Bot. **88**, 570-582.

West, M. A. L.; Harada, J. J.; 1993: Embryogenesis in higher plants: An overview. Plant Cell **5,** 1361-1369.

Zhu, Y. M.; Hoshino, Y.; Nakano, M.; Takahashi, E.; Mii, M.; 1997: Highly efficient system of plant regeneration from protoplasts of grapevine (*Vitis vinifera* L.) through somatic embryogenesis by using embryogenic callus culture and activated charcoal. Plant Sci. **123**, 151-157.

Received August 22, 2007