Botany Research International 3 (2): 48-55, 2010
ISSN 2221-3635

Induction of Somatic Embryogenesis and Plant Regeneration in Grapes (*Vitis vinifera* L.)

[1]*Ravindra B. Malabadi,* [2]*S. Vijaykumar,* [1]*K. Nataraja and* [1]*Gangadhar S. Mulgund*

[1]Department of Botany, Division of Plant Biotechnology, Karnatak University, Pavate nagar, Dharwad-580003, Karnataka state, India
[2]Department of Biotechnology, Madanpalle Institute of Technology and Science, Madanpalle-517325, Chitoor District, Andhra Pradesh, India

Abstract: This investigation highlights an *in vitro* protocol for callus induction, somatic embryogenesis and plant regeneration using leaf explants of three varieties (Thomson seedless, Sonaka and Tas-e-Ganesh) of grapevine. Leaf explants cultured on the full strength inorganic salts Nitsch and Nitsch [NN] basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ induced embryogenic tissue after 6-8 weeks. Somatic embryogenesis occurred in the presence of TDZ in combination with 2, 4-D, whereas it was never observed in explants cultured on control lacking hormones. The percentage of somatic embryogenesis was not similar in all the three tested varieties of *V. vinifera*. The highest percentage of somatic embryogenesis (78.0%) was recorded in case of Thomson seedless variety of *V. vinifera*, with a total of 55 somatic embryos recovered per gram fresh weight of embryogenic tissue. Out of 55 somatic embryos recovered, 43 somatic seedlings were established. This study indicates a high embryogenic potential of leaf explants of tested grapevine varieties and also opened up the possibility for large-scale clonal propagation of grapes.

Abbreviations: NN-Nitsch and Nitsch; 2,4-D-2, 4-dichlorophenoxy acetic acid; TDZ- Thidiazuron.
Key words: Grapevine · Clonal propagation · Leaf · Somatic embryogenesis

INTRODUCTION

Somatic embryogenesis is an experimental evidence where isolated somatic or body cells develop embryo-like structures under *in vitro* conditions by the influence of external growth hormones [1-10]. Plant regeneration from cultured cells is a prerequisite for the application of somatic techniques to crop improvement. This is a remarkable phenomena unique to plants only. The process is feasible because plants possess cellular totipotency whereby individual somatic cells can regenerate into a whole plant [11]. An advantage is also that the developmental process of the somatic embryos can be controlled and synchronised, allowing collection of embryos at specific stages. In addition to genetic transformation, somatic embryos are also promising material for germplasm cryopreservation and has been used in breeding strategies which exploit somaclonal variation [12].

Grapes are under large-scale cultivation in India particularly in Karnataka state. They are used for both table purpose and raisin making. Grapes are also susceptible to major fungal diseases and pests, hence, the growers incur huge expenditure on plant protection. Therefore, development of disease resistant grapevine varieties through the application of genetic engineering techniques is very much needed. Genetic improvement of grapevine by conventional breeding is impeded by long juvenile periods and inbreeding depression [13]. Therefore, embryogenic cultures are an attractive target for genetic transformation and the use of somatic embryogenesis would be of great benefit for genetic improvement of grapevine [14]. Somatic embryogenesis in *Vitis vinifera* cv. Cabernet-Sauvignon was first reported by Mullins and Srinivasan in 1976 for unfertilized ovules [15]. Subsequently, regeneration of *V. vinifera* somatic embryos has been reported from anthers [16-19], stigma and style [20], immature zygotic embryos [21], leaves

Corresponding Author: Ravindra B. Malabadi, Department of Botany, Division of Plant Biotechnology, Karnatak University, Pavate nagar, Dharwad-580003, Karnataka,

[22-23], tendrils [24], immature ovaries [25], as well as from filaments [26]. Nevertheless, considerable genotypic differences exist in grapevine somatic embryogenesis and results reported in the literature are varied [27]. Successful regeneration depends on many factors, including explant type, genotype, medium used and explant stage at the beginning of culture. The once limited number of *Vitis*-species and cultivars giving somatic embryos has recently increased. Somatic embryogenesis in grapes is hindered by low efficiency and strong genotype dependence, so there is not a general protocol for grape regeneration. However, the technique is not yet routine and improvements are needed in commercially important Indian grape genotypes. Here, we document the induction of somatic embryogenesis and regeneration of plantlets using leaf explants of *V. vinifera* L. cvs. Thompson seedless, Sonaka and Tas-e-Ganesh under the influence of TDZ as a growth regulator.

MATERIALS AND METHODS

Induction of Embryogenic Tissue: Leaf explants were harvested from 6-7- month- old mother plants of three *V. vinifera* L. cvs: Thompson, sonaka and Tas-e-Ganesh were carefully washed in double distilled water (DDW). They were surface decontaminated sequentially with 0.1% streptomycin (1 min), 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma-Aldrich, St. Louis, USA) and thoroughly rinsed with sterilized double distilled water. Leaf sections were cultured on Nitsch and Nitsch NN [28] basal medium with 3.0% sucrose, 0.7% agar, 0.5 gl^{-1} *myo*-inositol, 1.0 gl^{-1} casein hydrosylate, 0.5 gl^{-1} L-glutamine, 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid and 0.1 gl^{-1} biotin, all purchased from Sigma. The medium was supplemented with a range of thidiazuron (TDZ) concentrations (0.45, 2.27, 4.54, 9.08 and 11.35 μM) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) at a concentration of 4.52 μM singly and in combination without any other growth hormones. The cultures were raised in 25 mm × 145 mm glass culture tubes (Borosil, Mumbai, India) containing 15 ml of the above basal medium under cool white fluorescent light (Mysore lamps, India) at 100 μmol m^{-2} s^{-1} and 25 ± 3°C with a relative humidity of 55-60%. The pH of the media was adjusted to 5.8 with 1 N NaOH or HCl before agar was added. Media without TDZ and 2, 4-D served as the control. The media were then sterilized by autoclaving at 121°C at 1.04 Kg cm^{-2} for 15 min. L-glutamine, biotin, *p*-aminobenzoic acid and were filter sterilized (Whatman filter paper, pore size = 0.45 μm; diameter of paper = 25 mm) and added to the media after autoclaving when the medium had cooled to below 50°C.

All the cultures were examined for the presence of different developmental stages of somatic embryos by morphological and cytological observations of callus. The cultures showing oval, round, heart shaped embryos were identified and subcultured on the initiation medium for further 6 weeks for the better development of somatic embryos. The full strength inorganic salts NN [28] basal medium supplemented with 4.52 μM 2, 4-D and 4.54 μM TDZ (induction medium) was used as an effective induction medium for producing the embryogenic tissue. Embryogenic tissue showing different cell divisions such as 2 to 8 celled stages was identified using microscopic observation. On the other hand the callus without pro-embryonic cell divisions was considered as non-embryogenic. Non-embryogenic tissue was separated immediately from the rest of the tissue to avoid the overgrowth of the tissue. The efficiency of plant growth regulators and their concentrations were analyzed on the basis of visual observation (callusing percentage, percentage of explants forming embryogenic tissue, callus growth and callus necrosis). The ineffective treatments were discontinued.

Maintenance of Embryogenic Tissue: The embryogenic tissue was subcultured on the same induction media for every 4 weeks. All the cultures were maintained under a cool white fluorescent light (100μmol m^{-2} s^{-1}) at 25±3°C with a relative humidity of 55-60%. The percentage of cultures showing somatic embryogenesis has been recorded.

Somatic Embryo Maturation: The embryogenic tissue was transferred to maturation medium to induce cotyledonary embryo development. The full strength (inorganic salts) NN (Nitsch and Nitsch 1969) [28] basal medium supplemented with 3.0% sucrose, 5 μM ABA and 0.8% agar (maturation medium) was tested for this purpose. All the cultures were again maintained in the dark for 4 weeks. Microscopic observation was conducted to ensure the development of somatic embryos. The total number of somatic embryos produced after 8 weeks on maturation medium per one gram fresh weight of embryogenic tissue was recorded.

Germination and Recovery of Plantlets: After maturation, the cotyledonary somatic embryos were taken from the cultures for germination. The germination medium used was half strength (inorganic salts) NN (Nitsch and Nitsch 1969) [28] basal medium with 0.7% agar without any growth regulators (germination medium). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of an epicotyl. After 4 weeks on germination medium, the plantlets were directly transferred to vermiculite. Plantlets were placed in a growth room under a 16 hr photoperiod (50μ mol m^{-2} s^{-1}) for hardening. Somatic embryo proliferation in terms of root, shoot development, plant conversion was recorded.

Histological Preparation of Callus: The embryogenic tissue of *V. vinifera* with globular and heart shaped embryos were fixed in FAA (Formaldehyde solution-5ml, Glacial acetic acid-50ml, 90ml of 70% ethyl alcohol). After dehydrating them through ethanol-butanol series and embedding in paraffin, they were sectioned at 10μ thick [29]. The sections were stained with safranin and were made permanent by mounting them in DPX mountant for microscopic observation [29].

Statistical Analysis: In all the above experiments, each culture tube received a single explant. Each replicate contained 25 cultures and one set of experiment is made up of 2 replicates (50 leaf sections were cultured for one set of experiment for each *V. vinifera* cvs Thompson, sonaka and Tas-e-Ganesh). Nutrient medium without TDZ and 2, 4-D served as control. All the experiments were repeated 3 times. Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p<0.05$) or evaluated for independence using Chi-square test. Further, the differences contrasted using a Duncan's multiple range test (a=0.05) following ANOVA. All statistical analysis was performed using the SPSS statistical software package.

RESULTS AND DISCUSSION

In the present investigation, the leaf explants responded well and callus was induced after 2-4 weeks of culture on the full strength inorganic salts NN [28] basal medium supplemented with 4.52 μM 2, 4-D and 4.54 μM TDZ (induction medium) in all the three varieties of *V. vinifera*. The callus subsequently covered the entire surface of the leaf explants within 8 weeks. The intensity of callus proliferation was not similar in all the tested grapevine varieties. The primary callus was whitish but grew rapidly into yellowish brown friable callus on subsequent subculture. Better callus proliferation was found in these calli than in those that were watery and loose. Embryogenic areas were clearly visible from the rest of the callus by their globular and glazy appearance and emerged as distinct white structures (Figure-1B). In a control study, the leaf explants did not promote callus formation. Leaf explants remained green for two weeks and eventually turned brown and necrosed. Table 1 shows the embryogenic response of leaf-explants scored after 10 weeks of *in vitro* culture on the initiation medium. The highest percentage of induction of somatic embryogenesis (Thomson seedless-78.0%; Sonaka-56.2; Tas-e-Ganesh-85.0%) was observed on the full strength inorganic salts NN [28] basal medium supplemented with 4.52 μM 2, 4-D and 4.54 μM TDZ (induction medium) in all the three varieties of *V. vinifera* (Table-1; Figure-1A). Leaf explants failed to induce embryogenic tissue on the lower (0.45 μM) as well as on the higher (11.35 μM) concentrations of TDZ. Therefore, 4.52 μM 2, 4-D and 4.54 μM TDZ are the optimum concentrations for the induction of embryogenic tissue in all the 3 varieties of *V. vinifera*. The frequency of embryogenic tissue formation of three varieties of *V. vinifera* was varied from each other (Table-1). Success rates of the same cultivar in different years can differ, as many factors influence the differentiation of embryogenic callus. However, the trends are similar to those previously recorded, confirming differences in response among the cultivars tested. The embryogenic tissue was well maintained on the maintenance medium (Figure-1B). Microscopic observation of callus revealed different stages (2, 4 and 8 celled) of active cell division confirming the somatic embryogenesis on the maintenance medium in all the tested grapevine varieties (Figure-1C). Somatic embryogenesis occurred in the presence of TDZ in combination with 2,4-D, whereas it was never observed in explants cultured on control lacking hormones (Table 1). The presence of growth regulators was necessary to induce the regeneration of somatic embryos. In the present study, somatic embryos appeared to be fused at the base and sometimes subculture caused them to recallus on the maintenance medium. Therefore, it became necessary for the callus to be excised and subcultured

Table 1: The effect of various concentrations of TDZ on initiation of embryogenic tissue in three different varities of *V. vinifera* cultured 10 weeks on full strength NN basal medium supplemented with 4.52µM 2, 4-D and 4.54 µM TDZ

	Embryogenic tissue in 3 varieties *of V. vinifera* (%)		
TDZ (µM)	Thompson seedless	Sonaka	Tas-e-Ganesh
Control	0.0±0.0c	0.0±0.0c	0.0±0.0c
0.45	0.0±0.0c	0.0±0.0c	0.0±0.0c
2.27	18.0±0.3b	8.0±0.2 b	31.0±0.0b
4.54	78.0±1.9a	56.2±2.1a	85.0±1.4a
9.08	10.4±0.1b	4.0±5.02b	11.5±10.08b
11.35	0.0±0.0c	0.0±0.0c	0.0±0.0c

Control-1=NN basal medium without growth regulators such as 2, 4-D and TDZ

Data scored after 10 weeks and represents the mean ±SE of at least 3 different experiments. In each column, the values with different letters are significantly different (P<0. 5)

Table 2: Somatic embryogenesis and seedling recovery in three different varieties of *V. vinifera*

V. vinifera	Somatic embryogenesis (%)	Somatic embryos recovered per gram fresh wt of embryogenic tissue	Seedlings recovered per gram fresh wt of embryogenic tissue
Thompson Control	78.0±1.0a	55.0±2.1 a	43.0±0.1a
	0.0±0.0b	0.0±0.0b	0.0±0.0b
Sonaka Control	56.0±3.1a	40.0±3.0 a	31.0±0.4a
	0.0±0.0b	0.0±0.0b	0.0±0.0b
Tas-e-Ganesh Control	48.0±1.4a	23.0±2.9 a	20.0±0.2a
	0.0±0.0b	0.0±0.0b	0.0±0.0b

Control= NN basal medium without growth regulators

Data scored after 12 weeks and represents the mean ±SE of at least 3 different experiments

In each column, the values with different letters are significantly different (P<0. 05)

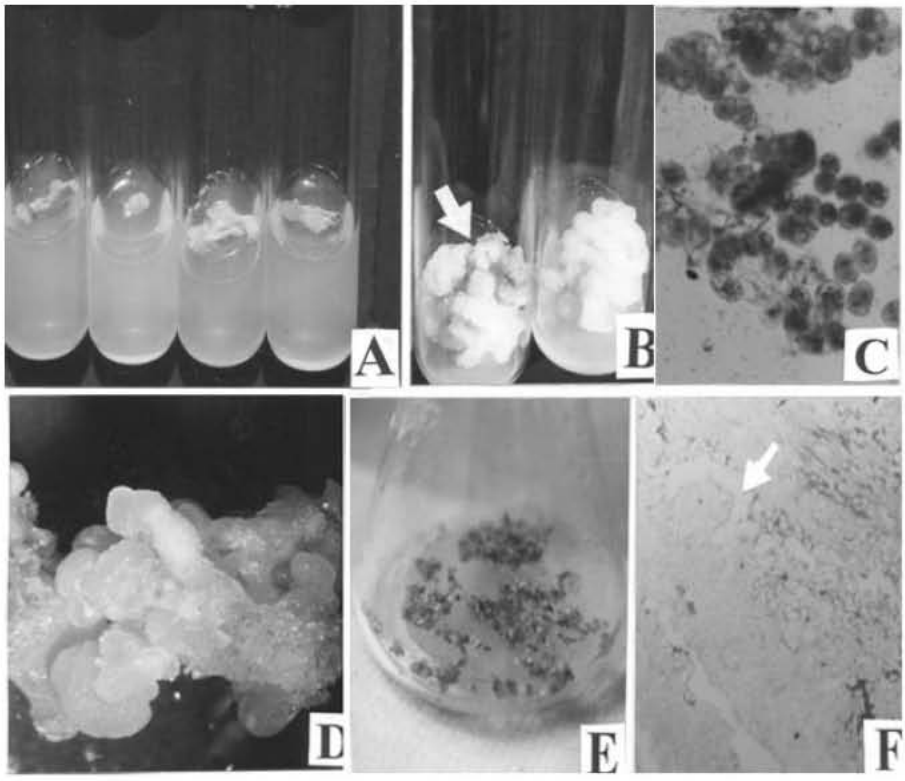

Fig. 1: TDZ induced somatic embryogenesis in *V. vinifera* (A)- Leaf explants showing proliferation of callus on initiation medium (10mm=3.80 mm). (B)-Proliferation of embryogenic callus on maintenance medium (10mm=7mm). (C)- Actively dividing cells with different stages of cell divisions seen under microscope (10mm=10.2mm). (D) Various developmental stages such as globular and oval shaped somatic embryos on maturation medium seen under the microscope. (E)-Germination of somatic embryos (10mm=2mm). (F) Histological preparation showing the differentiation of somatic embryos from the meristematic pocket.

along with the somatic embryos intact. The embryogenic tissue was subcultured on maturation medium for further development of somatic embryos. The full strength (inorganic salts) NN (Nitsch and Nitsch [28] basal medium supplemented with 3.0% sucrose, 5 μM ABA and 0.8% agar (maturation medium) was tested for this purpose. All the cultures were again maintained in the dark for 4 weeks. The total number of somatic embryos produced after 8 weeks on maturation medium per one gram fresh weight of embryogenic tissue is summarized in Table 2. Microscopic observation was conducted to ensure the development of somatic embryos. At first, somatic embryos appeared as small white to yellow protuberances on the surface of the callus. Somatic embryos were differentiated from the callus and not from the maternal tissue. In fact, we never observed embryogenesis without callus formation. It was also observed that frequency of somatic embryo formation was higher in white to yellowish, friable and crystal calli than in watery, loose ones. The embryos passed through recognizable globular, heart, torpedo and early cotyledonary stages, finally resulting in germinated embryos on the germination medium. Concomitant with morphological development, there was a change in color from white to green. The callus developed somatic embryos on maturation medium after a period of 4 to 6 weeks (Fig. 1D, F). The percentage of somatic embryogenesis was not similar in all the three varieties of *V. vinifera* (Table 2). The highest percentage of somatic embryogenesis (78.0%) was recorded in case of Thomson seedless variety of *V. vinifera*, with a total of 55 somatic embryos recovered per gram fresh weight of embryogenic tissue. Out of 55 somatic embryos recovered, 43 somatic seedlings were established in Thomson seedless variety of grapevine (Table 2). In case of Sonaka, 56.0% of somatic embryogenesis produced a total of 40 somatic embryos yielding 31 somatic seedlings per gram fresh weight of embryogenic tissue (Table 2). Lowest recovery of somatic embryos (23) was recorded with 48.0% of somatic embryogenesis in a Tas-e-Ganesh variety resulting in the recovery of 20 somatic seedlings (Table 2). Genotypic influence on somatic embryogenesis has been observed previously and a similar explanation holds for the differential response between the three cultivars tested by us. After maturation, somatic embryos were picked from the cultures for germination (Fig. 1E). Germination medium used was half strength (inorganic salts) NN medium with 0.7% agar without any growth regulators (germination medium). A large number of somatic embryos were continuously developed and germinated with a distinct shoot meristem and radicular end. Morphologically normal grapevine plants were transferred to *in vivo* conditions within 8 months of the initial culture.

TDZ is a substituted phenyl urea with cytokinin-like activity and therefore, stimulates rapid shoot differentiation [30]. TDZ aids in rapid plant regeneration of a number of plant species through organogenesis [31]. A protocol for induction of direct somatic embryogenesis, secondary embryogenesis and plant regeneration of *Dendrobium* cv. 'Chiengmai Pink' was developed using TDZ [32]. 5-25% of leaf tip segments of *in vitro*-grown plants directly formed somatic embryos on half-strength MS medium supplemented with 0.3, 1 and 3.0 mg/l TDZ [32]. Wilhelm reported successful micropropagation of juvenile Sycamore maple (*Acer pseudoplatanus*) *via* adventitious shoot formation by the use of 0.04 μM TDZ [33]. The rapid direct and repetitive somatic embryogenesis in *Coffea arabica* and *C. canephora* genotypes was tested on MS containing thidiazuron (TDZ) (1-phenyl-3-(1,2,3,-thiadiazol-5-yl)urea) in concentrations of 2.27–11.35s μM [34]. Segments taken from cotyledon leaf, first leaf and stalk of regenerated plantlets produced clusters of somatic embryos directly from cut portions of explants on TDZ (9.08 μM) containing medium within a period of two months. Subculturing of these embryo clusters produced more secondary embryos on reduced TDZ (0.045–0.91 μM) containing medium and these subsequently developed into plantlets (80–85%) on development medium followed by rooting on MS basal medium [34]. Recently Chhabra and coworkers reported that TDZ at concentration lower than 2.0 μM induced shoot organogenesis whereas at higher concentration (2.5-15 μM) it caused a shift in regeneration from shoot organogenesis to somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.) [35]. TDZ at 0.5 and 5.0 μM was found to be optimal for inducing an average of 4-5 shoots per cotyledonary node in 93 % of the cultures and 55 somatic embryos in 68 % of the cultures of lentil (*Lens culinaris* Medik.) [35]. The somatic embryos were germinated when transferred to lower TDZ concentration (0.5-1.0 μM) in lentil (*Lens culinaris* Medik.) [35]. In *Costus speciosus*, rhizome thin sections cultured on B_5 basal medium without TDZ (control) or with low concentrations 0.45 and 4.54 μM TDZ completely failed to produce shoot buds. Higher concentrations of TDZ, particularly 36.32, 40.86 and 45.41 μM, resulted in the browning of explants which finally necrosed [36]. On the other hand, initiation of shoot buds was observed in the range 11.35-27.34 μM TDZ with highest percentage

of rhizome thin sections (92%) producing shoot buds (12 ± 2.01) at 18.16 μM TDZ [36]. A sharp decrease in the number of shoot bud formation was also noticed when the concentration of TDZ was increased from 18.16 to 31.78 μM [36]. Least and poor growth of shoot buds (1.8 ± 0.02) was noticed at 31.78 μM TDZ in *Costus speciosus* [36]. An efficient shoot regeneration of *Eria dalzelli* (Dalz.) Lindl. for the first time was achieved using shoot tip tTCLs and TDZ [37]. As in this study, PLBs or proliferating shoot buds were observed when shoot tip tTCLs were cultured on the same basal medium supplemented with 9.08 μM TDZ. The highest percentage (96%) of PLBs survived and ultimately produced healthy shoots with 2-3 leaves [37]. An efficient shoot regeneration of *Vanda coerulea* was achieved using TCLs and TDZ [38]. PLBs or proliferating shoot buds was observed when thin shoot tip sections were cultured on Vacin and Went (1949, VW) basal medium supplemented with 11.35 μM TDZ. The highest percentage of PLBs (95%) survived and ultimately produced healthy shoots with 2-3 leaves when subjected to a 4-week TDZ treatment [38]. Treatment of soybean callus with TDZ stimulated cytokinin accumulation [39].

Singh and coworkers also reported TDZ-induced shoot multiplication in bamboo (*Dendrocalamus strictus*) and maximum number of shoots (14.8 ± 1.0) were obtained from shoot explants cultured in 2.27 μM TDZ supplemented half strength MS basal medium [40]. TDZ either alone (4.54 or 9.08 μM) or in combination with IAA (5.71 μM) on MS-supplemented medium induced a high frequency of shoot regeneration from primary leaf segments of three pigeonpea (*Cajanus cajan* L.) cultivars [41]. Very recently Chen and coworkers reported induction of totipotent callus on half strength MS medium supplemented with 22.60 μM 2,4-D and 4.54 μM TDZ in darkness in a *maudiae*-type slipper orchid, *Paphiopedilum* 'Alma Gavaert' [42]. The callus was proliferated more and maintained without any morphogenesis on the same medium over a 2-month interval. When callus was transferred to half-strength MS medium supplemented with 26.85 μM NAA, an average of 4.7 PLBs/shoot bud formed from each explant after 120 days of culture. On PGR-free basal medium, the percentage of explants with shoots was 33.3 and 0% and the 1 and 0 shoots/explant in hybrid PH59 and hybrid PH60, respectively [43]. The results of our experiments demonstrate the possibility for regeneration of plants *via* somatic embryogenesis from three seedless grapevine varieties cultured under *in vitro* conditions. This investigation has significant practical importance for further *in vitro* experiments. Obtaining of plants from inbreed seedless grapes and hybrids from crosses between seedless and seeded forms *via* somatic embryogenesis *in vitro* gives additional possibility to overcome the limitations and difficulties in the classical breeding programs of seedless grapes. Further, leaf explants, however, appear to be a novel source of expalnts for tissue culture of grape and hold great potential that needs to be exploited. A similar method could be followed to identify optimal stages for leaf culture in other important cultivars of *Vitis*. The somatic embryogenic lines have been used for genetic transformation and somatic hybridization of grapevines which are the most advanced techniques for genetic improvement of grape varieties.

REFERENCES

1. Konar, R.N. and K. Nataraja, 1965. Experimental studies in *Ranunculus sceleratus* L. Development of embryos from the stem epidermis. Phytomorphol., 15: 132-137.
2. Nataraja, K. and R.N. Konar, 1970. Induction of embryoids in reproductive and vegetative tissues of *Ranunculus sceleratus* L. *in vitro*. Acta Bot. Neerl., 19: 707-716.
3. Malabadi, R.B. and K. Nataraja, 2006a. Cryopreservation and plant regeneration *via* somatic embryogenesis using shoot apical domes of mature *Pinus roxburghii* Sarg. trees. *In vitro* Cell Deve Biol. Plant, 42: 152-159.
4. Malabadi, R.B. and K. Nataraja, 2006b. RAPD detect no somaclonal variation in cryopreserved cultures of *Pinus roxburghii* SARG. Prop. Orn. Plants, 6: 114-120.
5. Malabadi, R.B. and J. van Staden, 2005a. Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula*. Tree Physiol., 25: 11-16.
6. Malabadi, R.B. and J. van Staden, 2005b. Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. *In vitro* Cell Deve Biol. Plant, 41: 181-186.
7. Malabadi, R.B. and J. Van Staden, 2005c. Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. Plant Cell Tissue Organ Cult., 82: 259-265.
8. Malabadi, R.B. and J. Van Staden, 2006. Cold-enhanced somatic embryogenesis in *Pinus patula* is mediated by calcium. S. Afr. J. Bot., 72: 613-618.

9. Malabadi, R.B., J. Teixeira da Silva and K. Nataraja, 2008. A new approach involving salicyclic acid and thin cell layers for cloning mature trees of *Pinus roxburghii* (Chir Pine). The Am. J. Plant Sci. Biotech., 2(2): 56-59.
10. Malabadi, R.B., G.S. Mulgund and K. Nataraja, 2009. Triacontanol induced somatic embryogenesis and plantlet regeneration in *Catharanthus roseus*. J. Med. Arom. Plant Sci., 31: 147-151.
11. Reinert, J., 1959. Untersuchungen uber die Morphogenese an Gewebekulturen. Berichte der Deutschen Bptamoscjem Gesellschaft., 71: 15.
12. Martinelli, L. and I. Gribaudo, 2001. Somatic embryogenesis in grapevine (*Vitis* spp.). In: Roublekais-Angelakis, K., ed. Molecular biology and biotechnology of grapevine. Dordrecht: Kluwer., pp: 327-352.
13. Jayasankar, S., Z. Li. and D.J. Gray, 2000. *In vitro* selection of *Vitis vinifera* chardonnay with *Elsinoe ampelina* culture filterate is accompanied by fungal resistance and enhanced secretion of chitinase. Planta., 211: 200-208.
14. Antonio-Jose, Lopez-Perez., L. Velasco, M. Pazos-Navarro and M. Dabauza, 2008. Development of highly efficient genetic transformation protocols for table grape Sugraone and Crimson seedless at low *Agrobacterium* density. Plant Cell Tiss. Org. Cult., 94: 189-199.
15. Mullins, M.G. and C. Srinivasan, 1976. Somatic embryos and plantlets from an ancient clone of the grapevine (cv Cabernet-Sauvignon) by apomixes *in vitro*. J. Exp. Bot., 27: 1022-1030.
16. Mauro, M.C., C. Nef and J. Fallot, 1986. Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon. Plant Cell Rep., 5: 377-380.
17. Bouquet, A. and L. Torregrosa, 2003. Micropropagation of the grapevine (Vitis spp.). In: S.M. Jain, K. Ishi (Eds): Micropropagation of woody trees and fruits. Kluwer Academic Publishers, Dordrecht, The Netherlands, 75: 319-352.
18. Kikkert, J.R., M.J. Striem, J.R. Vidal, P.G. Wallace, J. Barnard and B.I. Reisch, 2005. Long-term study of somatic embryogenesis from anthers and ovaries of 12 grapevine (*Vitis* spp) genotypes. *In vitro* Cell. Dev. Plant-Biol., 41: 232-239.
19. Olah, R., A. Zok, A. Pedryc, S. Howard and L.G. Kovacs, 2009. Somatic embryogenesis in a broad spectrum of grape genotypes. Scientia Hortic., 120: 134-137.
20. Carimi, F., E. Barizza, M. Gardiman and F. Lo-Schiavo, 2005. Somatic embryogenesis from stigmas and styles of grapevine. *In vitro* Cell. Biol-Plant., 41: 249-252.
21. Stamp, J.A. and C.P. Meredith, 1988a. Proliferative somatic embryogenesis from zygotic embryos of grapevines. J. Am. Soc. Hort. Sci., 113: 941-945.
22. Stamp, J.A. and C.P. Meredith, 1988b. Somatic embryogenesis is from leaves and anthers of grapevine. Sci. Hort., 35: 235-250.
23. Das, D.K., M.K. Reddy, K.C. Upadhyaya and S.K. Sopory, 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.
24. Salunkhe, C.N., P.S. Rao and M. Mhatre, 1997. Induction of somatic embryogenesis and plantlets in tendrils of *Vitis vinifera* L. Plant Cell Rep., 17: 65-67.
25. Nakano, M., T. Sakakibara, Y. Watanabe and M. Mii, 1997. Establishment of embryogenic cultures in several cultivars of *Vitis vinifera* and *V X labruscana*. Vitis., 36: 141-145.
26. Nakajima, I. and N. Matsuta, 2003. Somatic embryogenesis from filaments of *Vitis vinifera* L. and *Vitis labruscana* Bailey. Vitis., 42: 53-54.
27. Rajasekaran, K. and M.G. Mullins, 1979. Embryos and plantlets from cultured anthers by hybrid grapevines. J. Exp. Bot., 30: 399-407.
28. Nitsch, J.P. and C. Nitsch, 1969. Haploid plants from pollen grains. Science., 163: 85-87.
29. Malabadi, R.B. and K. Nataraja, 2002. Peroxidase activity as a marker of xylogenesis in the cultured cells of *Psidium guajava* cv. Navaloor. Ind. J. For., 25(2): 196-200.
30. Mok, M.C., D.W.S. Mok., D.J. Armstrong, K. Shado, Y. Isogai and T. Okamoto, 1982. Cytokinin activity of N-phenyl N-1,2,3-thiadiazol-5-ylurea (thidiazuron). Phytochemistry, 21: 1509-1511.
31. Malik, K.A. and P.K. Saxena, 1992. Thidiazuron induces high frequency shoot regeneration in intact seedlings of pea, chickpea and lentil. Aust. J. Plant Physiol., 19: 731-740.
32. Chung, H.H., J.T. Chen and W.C. Chang, 2007. Plant regeneration through direct somatic embryogenesis from leaf explants of *Dendrobium*. Biol. Plantarum, 51: 346-350.
33. Wilhelm, E., 1999. Micropropagation of juvenile Sycamore maple via adventitious shoot formation by use of thidiazuron. Plant Cell. Tiss. Org. Cult., 57: 57-60.

34. Giridhar, P., V. Kumar, E.P. Indu, G.A. Ravishankar and A. Chandrasekar, 2004. Thidiazuron induced somatic embryogenesis in *Coffea arabica* L. and *Coffea canephora* P ex Fr. Acta Bot Croatia., 63(1): 25-33.
35. Chhabra, G., D. Chaydgart, M. Varma, M. Sainger and P.K. Jaiwal, 2008. TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.). Physiol. Mol. Biol. Plants., 14(4): 347-353.
36. Malabadi, R.B., G.S. Mulgund and K. Nataraja, 2004b. Thidiazuron induced shoot regeneration of *Costus speciosus* (Koen.) Sm using thin rhizome sections. S. Af. J. Bot., 70: 255-258
37. Malabadi, R.B., J.A. Teixeira da Silva and G.S. Mulgund, 2008a. Micropropagation of *Eria dalzelli* (Dalz.) Lindl. through TCL *in vitro* culture. Flor. Orn. Biotech., 2: 77-80.
38. Malabadi, R.B., G.S. Mulgund and K. Nataraja, 2004a. Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. Plant Cell. Tiss. Org. Cult., 76: 289-293.
39. Thomas, J.C. and S. Katterman, 1986. Cytokinin activity induced by Thidiazuron. Plant Physiol., 8: 681-683.
40. Singh, M., U. Jaiswal and V.S. Jaiswal, 2001. Thidiazuron – induced shoot multiplication and plant regeneration in bamboo (*Dendrocalamus strictus* Nees). J. Plant Biochem. Biotech., 10: 133-137.
41. Eapen, S., S. Tivarekar and L. George, 1998. Thidiazuron induced shoot regeneration in pigeonpea *(Cajanus cajan* L.). Plant Cell. Tiss. Org. Cult., 53: 217-220.
42. Chen, T.Y., J.T. Chen and W.C. Chang, 2002. Multiple shoot formation and plant regeneration from stem nodal explants of *Paphiopedilum* orchids. *In vitro* Cell. Dev. Biol – Plant, 38: 595-597.
43. Hong, P.I., J.T. Chen and W.C. Chang, 2008. Plant regeneration *via* protocorm-like body formation and shoot multiplication from seed-derived callus of a maudiae type slipper orchid. Acta Physiol. Plantarum, 30: 755-759.