CHAPTER 5:
SOMATIC EMBRYOGENSIS

5.1. Somatic embryogenesis

5.1.1. Introduction

Somatic embryogenesis has been defined as the initiation of embryos from plant somatic tissues closely resembling their zygotic counterparts. In nature, it is an asexual method of plant propagation that mimics many of the events of sexual reproduction. The sporophytic generation of a plant is initiated with a zygote, which is the initial cell (product of gamete fusion) that bears all the genetic information to develop an adult individual. In angiosperms, the zygote divides transversally, resulting in two cells. One of them, the apical cell, is small and dense with an intense activity of DNA synthesis. Further ordinal divisions of this cell give rise to the embryo head that will grow into a new plant. Development of somatic embryogenesis has been described to follow patterns similar to ones of zygotic embryogenesis (Altamura *et al*. 1992). Somatic embryogenesis can be induced artificially by the manipulation of tissues and cells *in vitro* potentially from almost any part of the plant body, directly from the explant or more frequently after a callus stage. Among the many factors involved in somatic embryogenesis, genotype is commonly considered as one of the most relevant. Other important factors include growth regulators, explant type and culture conditions.

In grape, somatic embryogenesis was first developed in 1970's with an aim to recover dihaploid plants for genetic improvement programs. In the first efforts, anthers were used for obtaining somatic embryogenesis (Gresshoff and Doy, 1974; Hirabayashi *et al*. 1976), and the first somatic embryos able to germinate were obtained from the hybrid Gloryvine (*V. vinifera* x *V. rupestris*) (Rajasekaran and Mullins, 1979). The first commercial planting of vines from somatic embryos was established in 1977 in Maryland, USA (Krul and Mowbray, 1984). Somatic embryogenesis in grapevines was achieved from nucellar tissues (Mullins and Srinivasan, 1976), leaves and petioles (Stamp and Meredith. 1988; Robacker, 1993, Martinelli *et al.,*1993; Bornhoff and Harst, 2000), tendrils (Salunkhe *et al.,*1997), zygotic embryos (Emershad and Ramming, 1994; Tsolova and Atanassov, 1994; Scorza, 1995), shoot apices (Barlass and Skene, 1978), anthers (Popescu, 1996; Salunkhe *et al.,*1999; Motoike *et al.,*2001; Perrin *et al.,*2004); stigma-style structure (Carmi *et al.,*2005), microspores (Sefc *et al.,*1997) and from immature anther and ovaries (Martinelli *et al.,*2001) but with lower frequencies and confined to only few genotypes. Due to high genotype dependence, some grape species and cultivars remain recalcitrant to the process of somatic

embryogenesis (Motoike *et al.,* 2001). Moreover, successful regeneration via somatic embryogenesis depends on the choice of explant, growth media and plant growth regulators (Bornholf and Harst, 2000). Thus, though there are a large number of reports available, however, the technique can not be said to be truly routine yet. Reports on somatic embryogenesis in grapes have been reviewed (Gray, 1995; Martinelli *et al.,* 2001). Somatic embryogenesis is the most utilized model system for plant totipotency and developmental studies and is one of the most powerful techniques offered by tissue culture for genetic improvement of plant species.

Embryogenic cultures have been reported to be highly suitable target materials for transformation studies in grapes due to high multiplication rates (Martinelli *et al.,* 1993; Perl *et al.,* 1995). Somatic and zygotic embryos exhibit a high degree of competence for repetitive somatic embryogenesis and plant regeneration. This idea has been exploited successfully in genetic transformation experiments, where whole somatic (Martineli *et al.,* 2000) and zygotic embryos (Scorza *et al.,* 1995) induced secondary embryogenesis after co-cultivation with *Agrobacterium* has been achieved.

5.1.2. Materials and methods

5.1.2.1. Plant material

In vitro leaves, petioles and internodes and *ex vitro* tendrils of Crimson Seedless were used as explants for induction of somatic embryogenesis. Leaves, petioles and internodes were collected from multiple shoot cultures maintained on full strength MS basal medium supplemented with BA (8.89 µM) and tendrils were collected from the mature vines growing at vineyard of the National Research Centre for Grapes (NRCG), Pune. Tendrils were surface sterilized and disinfected as per procedure described in the chapter 3.2.1.1. The explants were cut into 1 sq. cm pieces (in case of leaves) and 1cm long bits (in case of petioles, internodes and tendrils). Leaves were inoculated in petridishes with their abaxial (dorsal) surface in contact with the medium, while petiole, internodes and tendril explants were placed horizontally on the media.

5.1.2.2. Influence of growth regulators

Experiments were carried out to study the influence of different growth regulators on induction of somatic embryogenesis in *in vitro* leaves using half strength MS medium supplemented with BA (4.44 – 8.89), TDZ (4.54 – 9.08), 2,4-D (4.53 – 9.06), NOA (4.95 –

9.9), 3,6-Dichloro-o-anisic acid (DIC) (4.52 – 9.04), Picloram (PIC) (4.14 – 8.28) or 2,4,5-T (3.91 – 7.82).

5.1.2.3. Influence of BA and auxins

Based on our earlier results, experiments were carried out to improve the efficiency of somatic embryogenesis in Crimson Seedless. *In vitro* leaves were inoculated on half strength MS basal medium supplemented with BA (4.44) in combination with IAA (5.71) or NOA (4.95) or 2,4-D (4.53).

5.1.2.4. Influence of aminoacids

To further improve the efficiency of somatic embryogenesis in *in vitro* leaves, aminoacids were supplemented to half strength MS basal medium containing BA (4.44 μM) + NOA (4.95 μM). Aminoacids L-Glutamine, L-Cystein, L-Proline, L-Methionine, Phenylalanine and Arginine were supplemented in the medium at concentration of 2.5 and 5.0 μM.

5.1.2.5. Influence of explant type

Other explants of the cultivar were also tested for the induction of somatic embryogenesis. In two experiments, *in vitro* leaves and petioles; and internodes and tendrils were compared. Various treatments used for the study are described in Table 5.1.4 and 5.1.5.

As it is well known that success in somatic embryogenesis required an inductive treatment to initiate cell division and establish a new polarity in the somatic cell. Half strength MS basal medium supplemented with different plant growth regulators and aminoacids in varied concentrations were used for callus induction (Tables 5.1.1 – 5.1.5). After maintenance of the callus on respective callus induction media up to 4 months, the calli were shifted to hormone free half strength MS medium for the induction of somatic embryogenesis.

5.1.3. Results and Discussion

5.1.3.1. Influence of growth regulators

In the experiment carried out to investigate the influence of different plant growth regulators on induction of somatic embryogenesis in *in vitro* leaves of Crimson Seedless, it was found that all the explants cultured on ½ MS basal medium supplemented with either

TDZ, 2,4-D, 2,4,5-T, DIC, PIC or NOA induced profuse callusing. Type and intensity of callus varied among the treatments. Callus was white and compact in TDZ and semi compact to friable in case of 2,4-D or 2,4,5-T treatments. In case of BA treatments, the explants swelled at cut ends especially at proximal end of the leaf.

Explants cultured on half strength MS basal medium supplemented with BA (4.44 µM) induced somatic embryos in maximum number of explants *i.e.,* 22.22% followed by 20% in case of explants cultured in the media supplemented with BA (8.89 µM) or TDZ (9.08 µM) (Table 5.1.1). DIC treatments induced the highest number of embryos per explant. On an average, maximum 7.8 embryos per explant in presence of DIC (9.04 µM) followed by 6.7 embryos per explant in DIC (4.52 µM) were recorded.

Table 5.1.1: Influence of growth regulators on somatic embryogenesis in *in vitro* leaves of Crimson Seedless

Growth regulator (µM)	No. of explants inoculated	% of explants with callus	% of explants showing embryogenesis	Av. No. of embryos per explant
BA (4.44)	63	38.09	22.22	6.0
BA (8.89)	30	56.67	20.00	1.8
TDZ (4.54)	32	75.00	15.63	5.4
TDZ (9.08)	30	100.00	20.00	5.3
2,4-D (4.53)	32	100.00	6.25	6.0
2,4-D (9.06)	28	100.00	14.28	5.3
NOA (4.95)	30	100.00	6.67	5.5
NOA (9.9)	29	100.00	10.34	4.7
DIC (4.52)	32	100.00	18.75	6.7
DIC (9.04)	36	100.00	11.11	7.8
PIC (4.14)	35	100.00	8.57	6.0
PIC (8.28)	36	100.00	11.11	5.5
2,4,5-T (3.91)	35	100.00	5.71	7.0
2,4,5-T (7.82)	32	100.00	9.38	3.0
SEM±		5.10	1.82	
CD (p=0.01)		6.37	2.44	
		**	**	

*Basal medium – Half strength MS; ** Significant at 1% level

5.1.3.2. Influence of BA and auxins on the induction of somatic embryogenesis

Addition of auxin to the medium containing BA (4.44 µM) significantly improved the percentage of explants showing induction of callus and subsequent embryogenesis. Half strength MS medium supplemented with IAA (5.71 µM) induced maximum callogenic

response (100%), when used alone and 97.44% when supplemented with BA (4.44 µM). Percentage of explants showing somatic embryogenesis was maximum (30.56%) when NOA (4.95 µM) was added to the medium containing BA (Table 5.1.2). Number of embryos per responded explants increased up to 9.0 with the addition of 2,4-D to the medium along with BA (4.44 µM) compared to BA alone which induced 6.2 embryos / explant. Somatic embryogenesis response was lower on medium with auxin alone. Among auxins, NOA induced higher responses compared to other auxins tested.

Table 5.1.2: Influence of BA and auxins on somatic embryogenesis in *in vitro* leaves of Crimson Seedless

Growth regulators (µM)	No. of explants inoculated	% of explants showing callus	% of explants showing embryogenesis	Av. No. of embryos per explant
BA (4.44)	36	38.88	22.22	6.2
BA (4.44) + IAA (5.71)	36	97.44	19.44	7.0
BA (4.44) + NOA (4.95)	36	88.89	30.56	7.2
BA (4.44) + 2,4-D (4.53)	35	94.26	22.86	9.0
IAA (5.71)	35	100.00	11.43	4.0
NOA (4.95)	36	88.89	5.56	6.0
2,4-D (4.53)	34	100.00	8.82	3.3
SEM±		9.05	3.37	
CD (p=0.01)		11.11	3.68	
		**	**	

*Basal medium – Half strength MS; ** Significant at 1% level

Induction of somatic embryogenesis on medium supplemented with 2,4-D has been reported for *Vitis* by different workers (Robacker, 1993) using leaf, petioles and anthers. 2,4-D was found to be very effective in inducing somatic embryogenesis (Gray and Meredith, 1992) but inhibited subsequent embryo development (Komamine, 1992) in grapevine. Salunkhe *et al*. (1999) observed that an initial exposure of 2,4-D was essential to trigger the embryogenic potential and subsequent embryo production in anthers of *Vitis latifolia* L.

5.1.3.3. Influence of aminoacids

An additional supplement of aminoacids to the medium used for callogenesis substantially improved the efficiency of somatic embryogenesis in *in vitro* leaves of Crimson Seedless. All the amino acids induced callogenesis in leaves, though percentage of response

varied significantly. Increase in concentration of aminoacid from 2.5 to 5.0 µM had no improvement in the response. Phenylalanine (5.0 µM) was the most effective amino acid inducing somatic embryogenesis in the highest number of explants (55.26%) with an average of 5.5 embryos/explant (Table 5.1.3).

Table 5.1.3: Influence of aminoacids on somatic embryogenesis in *in vitro* leaves of Crimson Seedless

Amino acid concnetration (mM)	No. of explants inoculated	% of explants showing callus	% of explants showing embryogenesis	Av. No. of embryos per explant
Control	40	100.0	30.00	2.4
Glutamine (2.5)	36	100.0	44.44	4.7
Glutamine (5.0)	36	100.0	44.44	3.7
L-Cystein (2.5)	36	100.0	41.67	4.5
L-Cystein (5.0)	36	100.0	47.22	3.2
Proline (2.5)	40	100.0	45.00	4.7
Proline (5.0)	40	100.0	45.00	3.8
Methionine (2.5)	40	100.0	35.00	4.9
Methionine (5.0)	40	100.0	30.00	4.9
Phenylalanine (2.5)	39	100.0	53.85	4.6
Phenylalanine (5.0)	38	100.0	55.26	5.5
Arginine (2.5)	38	100.0	42.11	4.3
Arginine (5.0)	39	100.0	46.15	4.1
SEM±		0.50	2.96	-
CD (p=0.01)		1.15	6.51	-
		**	**	-

*Basal medium used – Half strength MS + BA (4.44 µM) + NOA (4.95 µM)
** Sidnificant at 1% level

Aminoacids are building blocks of the proteins and they are known to play a key role in different plant metabolic processes. Amino acids have influence on various plant mechanisms like DNA replication, RNA synthesis and protein metabolisms there by influencing cell growth and cell division. Addition of aminoacids to medium was reported to be beneficial for the induction of embryos and plant regeneration in *T. aestivum* (Trottier *et al.,*1993), *H. vulgare* (Ouédraogo *et al.,*1998) and *Cucumis sativus* L. (Ashok Kumar *et al.,*2003). Addition of alanine, asparagine or glutamine to embryo induction medium

increased the frequency of embryo differentiation and the percentage of plant regeneration in *H. vulgare* (Muyuan *et al.,* 1990). Ashok Kumar *et al*. (2003) reported that addition of a combination of aminoacids to induction medium enhanced both embryogenesis and plantlet regeneration in two cultivars of *Cucumis sativus* L.

5.1.3.4. Influence of explant type

Between the *in vitro* leaf and petiole explants, petioles induced higher responses with respect to callus and subsequent embryogenesis at similar growth regulator treatments. In case of leaves, callusing was observed along the cut surfaces, and midrib and sometimes on the entire leaf depending on the treatment. Mostly the callus was loose and friable (Fig. 5.1.1A). Whereas, semi friable to compact callus was observed at cut ends of the petioles near to petiolar stub (Fig. 5.1.1B).

Over all, callogenesis was 76.6% in petiole explants and 75.0% in leaf explants (Table 5.1.4) (Fig. 5.1.2A). The highest callogenesis response (100.0) in both the explant types was observed in case of leaves cultured in medium with NAA (1.07 µM) alone or supplemented with TDZ (4.54 µM - 9.08 µM) and lowest in KIN (9.3 µM).

Medium supplemented with TDZ (9.08 µM) and NAA (1.07 µM) induced the maximum embryogenic response of 36.1 % and 34.3 % in leaf and petiole explants, respectively. The average number of somatic embryos was 7.3/explant in case of petiole and 6.1/explant in case of leaf explants. Supplement of NAA (1.07 µM) alone did not affect any embryogenesis response in both the explants.

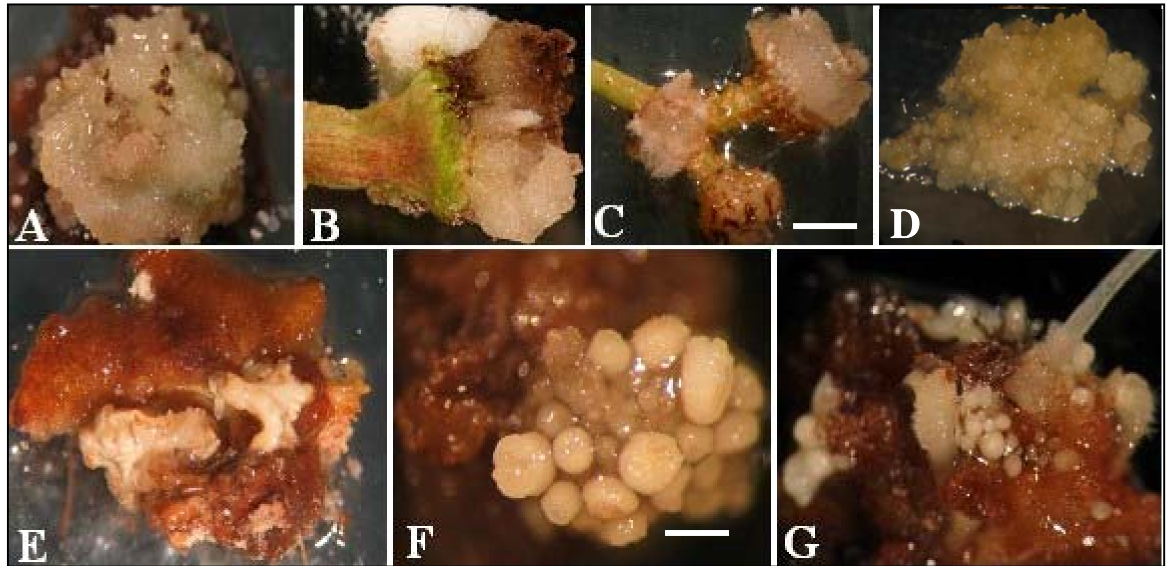


Fig. 5.1.1. Somatic embryogenesis from explants of Crimson Seedless. Callogenesis from leaf (A), petiole (B) and tendril (C), embryogenic callus (D), somatic embryos from dorsal surface of leaf (E&F) and petiole (G) Bar = 250 μm

Table 5.1.4: Influence of growth regulators on somatic embryogenesis in *in vitro* leaf and petiole explants of Crimson Seedless

Growth regulators (µM)	No. of explants inoculated		% of explants showing callus		% of explants showing embryogenesis		Av. No. of embryos per explant	
	Leaves	Petioles	Leaves	Petioles	Leaves	Petioles	Leaves	Petioles
KIN (4.65) + NAA (1.07)	36	36	83.33	88.89	16.67	30.56	4.0	6.5
KIN (9.3) + NAA (1.07)	36	36	86.11	91.67	13.89	22.22	6.4	7.5
Zeatin (4.56) + NAA (1.07)	35	34	82.86	85.29	11.43	17.65	3.5	6.0
Zeatin (9.12) + NAA (1.07)	36	30	80.56	96.67	13.89	23.33	5.4	6.3
TDZ (4.54) + NAA (1.07)	36	35	100.00	91.43	27.78	34.29	6.9	7.6
TDZ (9.08) + NAA (1.07)	36	36	100.00	94.44	36.11	36.11	7.1	8.6
KIN (9.3)	36	34	5.56	5.88	2.78	2.94	7.0	9.0
Zeatin (9.12)	36	35	22.22	22.86	2.78	5.71	10.0	8.5
TDZ (9.08)	36	36	91.67	94.44	19.44	16.67	5.9	6.7
NAA (1.07)	33	34	100.00	94.11	0	0	0	0
Total / Mean	356	346	75.0	76.6	14.6	19.1	6.1	7.3
SEM±			6.69	5.92	3.63	3.90		
CD (p=0.01)			10.60	10.47	4.54	6.72		
			**	**	**	**		

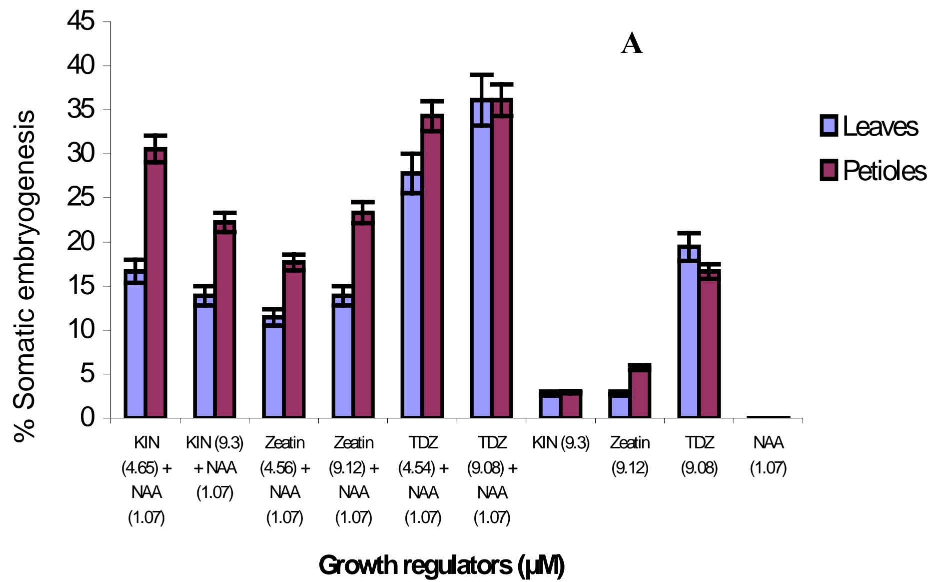
*Basal medium – Half strength MS, ** Significant at 1% level

Due to sterility and availability of materials round the year independent of seasonal variations, explants like leaf, petiole and internode derived from *in vitro* cultures were used in the present study. Tendrils from field grown vines of Crimson Seedless were available during flowering period. Callogenic response varied from zero in hormone free medium to the maximum of 100% in most of the treatments (Table 5.1.5). Callus was semi compact to compact seen at cut ends and injured portions mostly (Fig. 5.1.1C). Auxins were found to be superior in inducing callus followed by TDZ. Percentage of explants with embryogenesis was maximum (38.9%) in case of tendrils cultured in the medium supplemented with TDZ (4.0 μM) + NOA (20.0 μM) followed by 32.5% in the medium supplemented with TDZ (10.0 μM) + 2,4,5-T (10.0 μM) (Table 5.1.5) (Fig. 5.1.2B). Medium with BA (10.0 μM) + NAA (0.4 μM) induced the least response (7.5%) in both the explants. Between tendrils and internodes, tendrils induced higher callogenesis and embryogenesis responses. Medium supplemented with TDZ (10.0 μM) + 2,4,5-T (10.0 μM) induced embryogenesis in 25% of internode explants. Average number of embryos induced per responded explant varied from 5.5 in the medium supplemented with BA (10.0 μM) + 2,4,5-T (10.0 μM) to 8.0 in the medium supplemented with BA (10.0 μM) + NAA (0.4 μM). As reported earlier (Perrin *et al*. 2004) there was no correlation between frequency of callogenesis and somatic embryogenesis in the present study. Compared to other explants, tendrils induced higher callogenesis and embryogenic responses.

Table 5.1.5: Influence of growth regulators on somatic embryogenesis in tendril and internode explants of Crimson Seedless

Growth regulators (µM)	No. of explants inoculated		% of explants showing callus		% of explants showing embryogenesis		Av. No. of embryos per explant	
	Tendril	**Internode**	**Tendril**	**Internode**	**Tendril**	**Internode**	**Tendril**	**Internode**
BA (10.0) + 2,4-D (10.0)	39	35	100.00	98.5	17.95	11.43	7.0	6.3
BA (10.0) + 2,4,5-T (10.0)	40	36	95.00	89.2	20.00	19.44	5.5	5.1
TDZ (10.0) + 2,4-D (10.0)	38	37	100.00	96.2	26.32	13.51	5.9	7.8
TDZ (10.0) + 2,4,5-T (10.0)	40	32	100.00	98.2	32.50	25.00	7.1	7.0
BA (20.0) + NAA (10.0)	40	40	92.50	85.5	7.50	10.00	8.0	7.3
BA (20.0) + NOA (10.0)	34	40	88.24	76.5	17.65	12.50	6.0	11.2
BA (40.0) + NAA (20.0)	36	36	94.57	90.5	10.25	11.11	6.3	6.3
BA (40.0) + NOA (20.0)	32	40	100.00	94.5	28.13	20.00	6.0	5.6
TDZ (2.0) + NAA (10.0)	39	36	99.67	95.7	11.20	11.11	6.5	5.3
TDZ (4.0) + NAA (20.0)	40	36	100.00	100.0	18.33	11.11	4.9	7.8
TDZ (2.0) + NOA (10.0)	36	36	100.00	95.5	30.56	16.67	7.2	9.6
TDZ (4.0) + NOA (20.0)	36	35	100.00	95.6	38.89	22.86	7.9	10.6
SEM±			1.74	1.68	2.85	1.59		
CD (p=0.01)			5.03	4.56	3.27	3.33		
			**	**	**	**		

* Basal medium – Half strength MS, ** Significant at 1% level



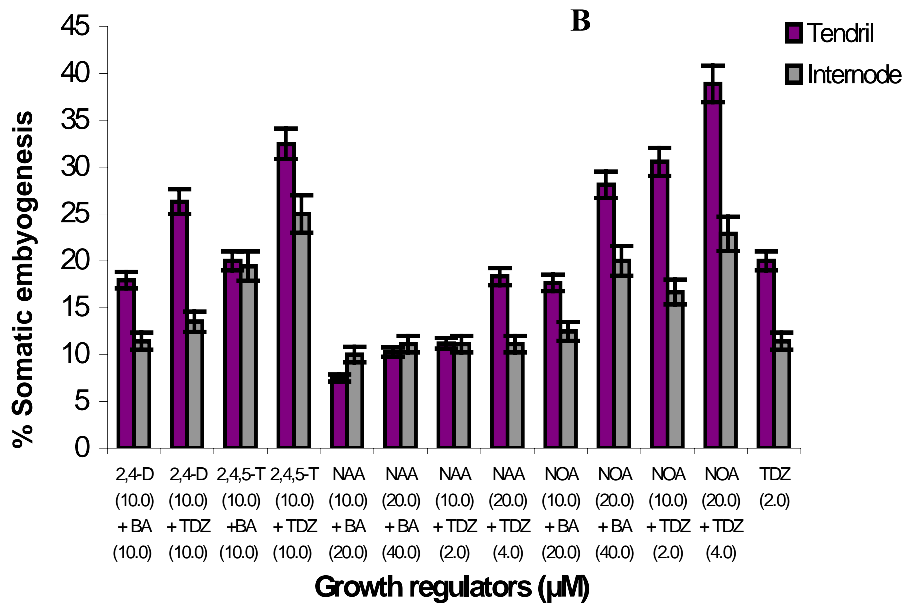


Fig. 5.1.2. Somatic embryogensis in Crimson Seedelss. A: Percent explants showing somatic embryogenesis from leaf and petioles and B: Percent explants showing somatic embryogenesis from tendril and internodes.

Our strategy for induction of somatic embryogenesis was based on concept of two steps method (Franks *et al*. 1998). In the first step, induction and proliferation of callus was achieved. While in the second step, development of embryogenic callus and induction of somatic embryos was achieved on differentiation medium. Where, compact white callus changed into granular in texture (Fig. 5.1.1D) and developed somatic embryos.

5.1.4. Conclusion

Somatic embryogenesis, widely considered to have a single cell origin, is particularly advantageous in studies on spontaneous variation, which originates during *in vitro* culture or experimentally induced by mutagens or transformation (Chen *et al.,* 1999). Present study demonstrates that somatic embryogenesis could be successfully induced from various explants of the cultivar and the efficiency of the protocol makes it highly useful for genetic transformation studies of the cultivar.

5.2. Zygotic embryo recovery

5.2.1 Introduction

Grape improvement programmes have been aimed to develop new seedless cultivars of good quality, high yield and large berry size (Emershad and Ramming, 1984; Bouquet and Davis, 1989). Conventional hybridization to obtain seedless progenies using seeded cultivars as female parents is of limited use due to a low proportion of seedless progeny (<15%) (Spiegel-Roy *et al.,* 1990; Singh and Brar, 1992). In seedless table grapes, fertilization takes place but embryo and/or endosperm development stops soon after anthesis and the seed aborts in different stages of development depending on the cultivar (Winkler *et al.,* 1974; Bouquet and Davis 1989). This phenomenon of embryo abortion is called stenospermocarpy. By *in vitro* embryo rescue technique, it is possible to rescue the embryos and obtain seedlings (Cain *et al.,* 1983; Emershad and Ramming 1984; Spiegel-Roy *et al.,* 1985; Gray *et al.,* 1987; Tsolova, 1990; Bharathy *et al.,* 2003 and 2005). Earlier, seedless cultivars could only be used as pollen parents but now with ovule/embryo culture technique, it is now possible to use seedless vines as female parents too (Cain *et al.,*1983; Emershad and Ramming, 1984; Gray *et al.,*1987; Emershad *et al.,*1989) in grape breeding programmes. *In-ovulo* embryo culture of stenospermocarpic grapes was used to obtain higher frequency of plant production (Fernandez *et al.,* 1991) and to obtain triploid grape seedlings from tetraploid and diploid cultivars (Yamashita *et al.,* 1993 and 1995). The success in plant production from cultured embryos largely depends on the stage of maturation of embryos and composition of the culture medium (Sharma *et al.,* 1996).

There are very few reports on influence of pre-bloom sprays of plant growth regulators so far. These include positive influence of BA on embryo recovery in crosses involving Thompson Seedless and Flame Seedless as female parents (Bharathy *et al.,* 2003 and 2005), improved seed germination with pre-bloom sprays of XE 1019 (Terbutrazole) in the cultivar C35-33 (Ledbetter and Shonnard 1990) and increased ovule number per berry, embryo recovery and plantlet production in grapevine cvs. CG 101.011 and Malvinas with pre-bloom spray of CCC (Aguero *et al.,* 1995).

5.2.2. Materials and methods

5.2.2.1. Influence of berry age

This experiment was carried out to optimize the age of berries for the maximum recovery of ovules and zygotic embryos. For this purpose, berries of Crimson Seedless were collected at 35, 45 and 55 d post anthesis from field grown vines at NRCG.

5.2.2.2. Influence of CPPU spray and BA in the culture medium

Another experiment was carried out to evaluate the influence of pre-bloom sprays on ovule / embryo recovery. First spray of N-(2-Chloro-4-pyridyl)-N'-phenylurea (CPPU) at 1 ppm was given to parrot green stage panicles i.e. about 10 d prior to flowering and second spray after 7 d of the first one. Berries (Fig. 5.2.3A) were collected at 55 d post anthesis and pre-chilled at 4°C for one week. After that berries were surface sterilized and disinfected as per the procedure described in chapter 3.2.1.1.

Method of ovule / embryo culture was followed as reported by Bharathy *et al.* (2003). After disinfection treatment, berries were blotted dry on sterile filter paper and ovules were excised from the berries aseptically. The excised ovules were cultured on ER (Emershad and Ramming, 1984) medium supplemented with BA at different levels (0.44–44.44 µM) ± GA_3 (2.89) and sucrose (6%) (Fig. 5.2.1B). After 60 d of culture, embryos were excised from ovules aseptically and cultured on Woody Plant medium (Llyod and McCown, 1981) supplemented with BA (0.89 µM). Observations such as number of ovules / embryos recovered from each treatment after 60 d of inoculation and the numbers of embryos geminated after 30 d of culture were recorded.

5.2.2.3. Secondary somatic embryogenesis and germination of somatic embryos

Somatic embryos were cultured on hormone free half strength MS basal medium for induction of secondary embryos or germination. Well formed somatic embryos were tested for germination on different basal media *viz.,* NN, WPM or ½MS supplemented with BA (0.89 – 4.44 µM) ± IBA (0.049 – 0.49 µM). Sucrose (3 %) was added to all the media gelled with agar (0.65%). All the media were autoclaved at 121°C and 15 psi for 20 min.

Germinated embryos were transferred to plastic cups containing a mixture of soil, sand and coco-peat (1:1:1) and plantlets were hardened as per procedure described in the chapter 2.14.

5.2.3. Results and Discussion

5.2.3.1 Influence of berry age

Berry size in terms of length and breadth gradually increased from 35 to 55 d post anthesis. Average berry weight increased from 400 mg at 35 d to 869.5 mg at 55 d (Table 5.2.1). Average ovule weight increased with the berry age, but had no influence on ovule recovery. The maximum average ovule weight (5.9 mg) was recorded at 45 d post

anthesis. Embryo recovery had positive correlation with berry age and size but not with ovule size. The maximum embryo recovery (4.95%) was achieved from berries collected at 55 d post anthesis.

Table 5.2.1: Influence of age of berries on embryo recovery in Crimson Seedless

Age of berries (days after anthesis)	Berry size		Av. berry weight (mg)	Av. No of Ovules recovered / berry)	Av. ovule weight (mg)	Embryo recovery (%)
	Length (cm)	Width (cm)				
35	11.26	7.99	400.0	2.59	5.5	2.41
45	11.97	8.69	472.8	2.58	5.9	4.76
55	14.12	10.21	869.5	2.57	5.8	4.95
SEM±			11.1	0.05	0.01	0.11
CD (p=0.01)			19.0	0.08	0.02	0.19
			**	NS	**	**

** Significant at 1% level

Increase in embryo recovery with age of berry could be an influence of ovule maturity as reported by Bouquet and Davis (1989). Tsolova (1990) observed that ovules at 52 d post anthesis gave the maximum embryo recovery compared to 66 d. In other reports, embryo recovery has been tested using berries from 10 d to 100 d post anthesis and found that berries at 40 to 60 d resulted in the higher recoveries (Cain *et al.,* 1983; Spiegel-Roy *et al.,* 1985; Emershad *et al.,* 1989; Gray *et al.,* 1990).

5.2.3.2. Influence of CPPU sprays and BA concentration in the medium

Pre-bloom sprays of CPPU had significant influence on berry and ovule parameters in addition to embryo recovery (Table 5.2.2). Berry size and weight varied marginally with CPPU sprays. Berry weight increased with CPPU sprays. Foliar application of CPPU was reported to accelerate the fruit growth in Watermelons (Kano, 2000) and increase berry weight in Kyoho grapes (Han and Lee, 2004). In our study, average berry weight was higher (869.3 mg) in CPPU sprayed treatments. Ovule recovery had negative correlation with CPPU sprays. Ovule recovery decreased with CPPU sprays. There was no correlation between ovule recovery and ovule weight. However, in several other studies ovule size had influence on % embryo recovery in different grapevine cultivars (Cain *et al.,* 1983; Burger and Goussard, 1996; Goldy and Amborn, 1987; Spiegel-Roy *et al.,* 1990).

Table 5.2.2: Influence of pre-bloom sprays of CPPU on ovule recovery in Crimson Seedless

Treatment	Av. berry weight (mg)	Av. No. of ovules / berry	Av. ovule weight (mg)
Control	515.3	2.57	10.81
CPPU Spray	869.3	2.57	5.82
SEM±	9.72	0.09	0.95
CD (p=0.01)	16.84	0.16	1.64
	**	**	**

*Basal medium used – Emershad and Ramming medium, ** Significant at 1% level

Pre-bloom sprays of CPPU and inclusion of BA in the ovule culture medium improved the over all embryo recovery. In earlier reports from our laboratory, pre-bloom sprays of BA were found to have a positive influence on embryo recovery and germination in hybrids of Thompson seedless (Bharathy *et al.* 2003) and Flame seedless (Bharathy *et al.* 2005), when the above two cultivars were used as female parents. Addition of growth regulators in the ovule culture medium significantly improved the percentage of embryo recovery compared to control. Percentages of embryo recovery were 22.04 and 17.06 from ovules cultured in the medium supplemented with BA (0.89 μM) and (2.22 μM), respectively. Medium devoid of PGR (control) resulted in 7.32 % of embryo recovery (Table 5.2.3). The beneficial effects of growth regulators especially BA, IAA and GA_3 in enhancement of embryo recovery in cultivars of grapevine have earlier been reported (Tsolova, 1990; Burger, 1992). In the present study, embryo recovery increased with the increase in BA concentration up to 0.89 μM but decreased on higher BA concentrations. Addition of GA3 did not have any positive effect on embryo recovery both in control and CPPU treatments.

In the present study, a synergistic effect of CPPU sprays and BA in culture medium on embryo recovery was observed. The percentage of embryo recovery depended on CPPU sprays and concentration of BA in the medium (Table 5.2.3). Cytokinins play an important role in stimulating both cell division and cell enlargement as well delay of tissue senescence and fruit ripening. It is reported that cytokinins show activity till 4 weeks after anthesis in developing seeds, disappear during fifth week and remains absent till ripening of berries (Pandey, 1982). The pre-bloom sprays of CPPU that has cytokinin-like properties and inclusion of BA in the medium may have overcome the deficiency of cytokinins, which eventually led to better ovule and embryo development. Cytokinins are assumed to establish seed as sink for assimilates for regulating cell division, initially in the ovary and subsequently in the meristem of the

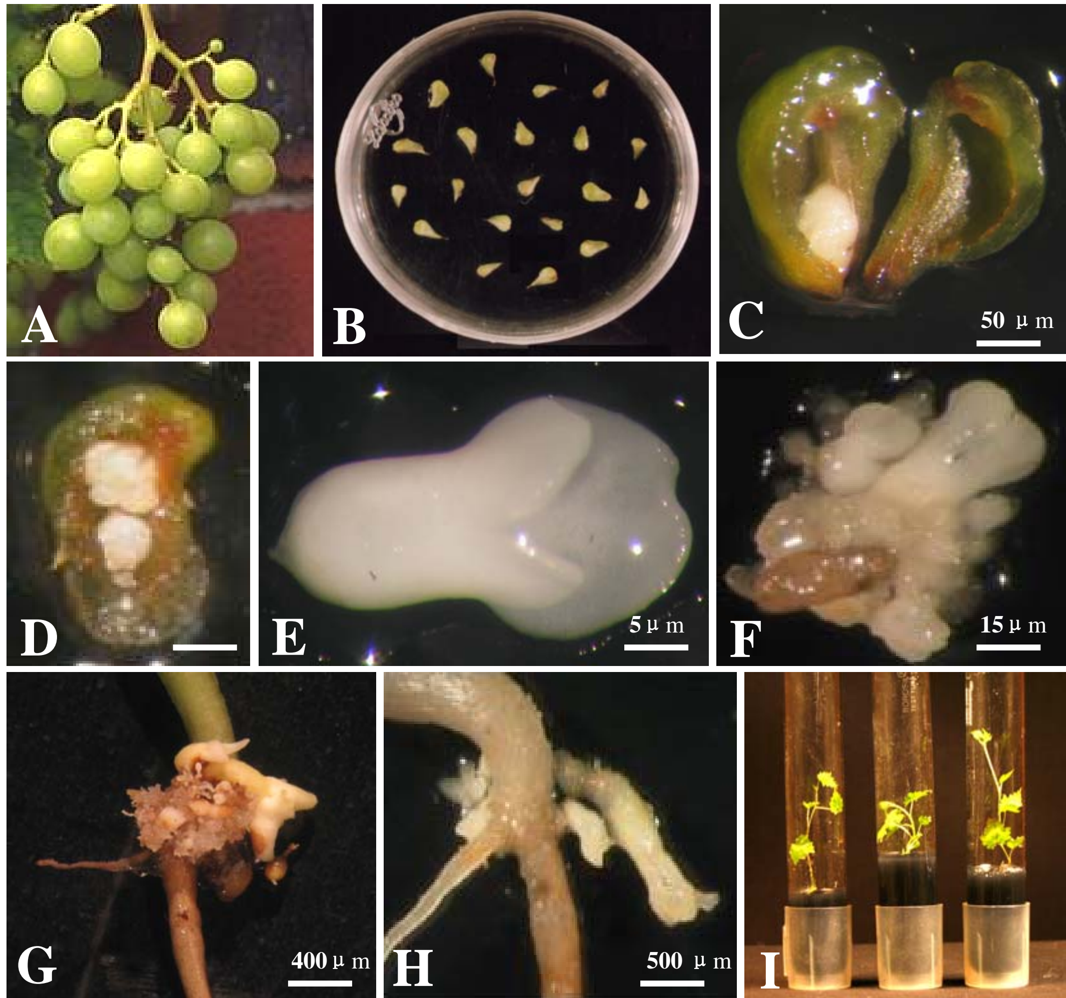


Fig. 5.2.1. Stages of zygotic embryo recovery in Crimson Seedless. A: immature berries, B: excised ovules, C & D: zygotic embryos inside ovules, E: recovered embryo, F: Somatic embryogenesis, G & H: Somatic embryogenesis from hypocotyl–radicle junction and I: Zygote derived plants of Crimson Seedless. Bar = 5 μ m

embryos hence are required for seed development (Atkins *et al.,* 1998). Thus, it is conceivable that exogenous supply of CPPU in form of pre-bloom sprays and inclusion of BA in the medium during ovule / embryo culture may enhance sink strength of these organs and result in the higher embryo recovery.

Table 5.2.3: Influence of pre-bloom sprays of CPPU and culture medium on embryo recovery in Crimson Seedless

<table>
<tr><th>Growth regulators (µM)</th><th colspan="2">Control</th><th colspan="2">CPPU Sprayed</th></tr>
<tr><td>Control</td><td colspan="2">2.00</td><td colspan="2">3.50</td></tr>
<tr><td>BA (0.44)</td><td colspan="2">5.87</td><td colspan="2">8.34</td></tr>
<tr><td>BA (0.89)</td><td colspan="2">8.33</td><td colspan="2">9.40</td></tr>
<tr><td>BA (2.22)</td><td colspan="2">6.00</td><td colspan="2">7.92</td></tr>
<tr><td>BA (4.44)</td><td colspan="2">4.22</td><td colspan="2">7.33</td></tr>
<tr><td>BA (8.89)</td><td colspan="2">3.17</td><td colspan="2">6.38</td></tr>
<tr><td>BA (22.22)</td><td colspan="2">2.00</td><td colspan="2">4.66</td></tr>
<tr><td>BA (0.89) + GA_3 (2.89)</td><td colspan="2">4.00</td><td colspan="2">8.00</td></tr>
<tr><td>BA (2.22) + GA_3 (2.89)</td><td colspan="2">3.33</td><td colspan="2">8.56</td></tr>
<tr><td>GA_3 (2.89)</td><td colspan="2">1.00</td><td colspan="2">4.66</td></tr>
<tr><td></td><td>Spray (S)</td><td colspan="2">Medium (M)</td><td>S X M</td></tr>
<tr><td>SEM±</td><td>0.16</td><td colspan="2">0.34</td><td>0.48</td></tr>
<tr><td>CD (p=0.01)</td><td>0.27</td><td colspan="2">0.59</td><td>0.84</td></tr>
<tr><td></td><td>**</td><td colspan="2">**</td><td>**</td></tr>
</table>

*Basal medium used – Emershad and Ramming medium, ** Significant at 1% level

Primary physiological effects of CPPU on grapevines involve the regulation of fruit set, berry growth and development. In earlier studies, CPPU has been shown to stimulate higher fruit yields in grape (Intrieri *et al.,* 1992; Zabadal and Bukovac, 2006), apple (Greene, 1989), cranberry (Devlin and Koszanski, 1988). Also CPPU stimulated fruit set when applied at or just before flowering in kiwifruit (Patterson *et al*. 1993). Increased embryo development in grapevine influenced by addition of BA in the medium (Gray *et al.,* 1990) and pre-bloom sprays (Bharathy *et al.,* 2003 and 2005) has earlier been reported. However to best of our knowledge, there is no report on combined effect of CPPU and BA on embryo recovery in grapevine.

Dissection of the ovules revealed that most of them were hollow and contained no visible embryo or endosperm remnants even when their outer integument tissues remained green. Callus was produced by the outer layers of the outer integument of the ovules. Ovules those failed to enlarge revealed a hollow space inside on dissection. Polyembryos and secondary embryos obtained from one ovule were regarded as one embryo to interpret the results. Polyembryony was observed in very few cases especially in case of ovules cultured in the medium supplemented with BA (0.89 µM). Polyembryony was earlier reported in cultured ovaries of *Anethum* (Johri and Sehgal, 1963), *Ribes* (Zatyko *et al*., 1981), Citrus (Kochba *et al*., 1972) and *Vitis* (Srinivasan and Mullis, 1980) and in matured seeds of *Vitis* (Bouquet, 1982). It might be assumed that inclusion of higher doses of vitamins and hormones in the medium was responsible for the production of higher percentage of polyembryony (Tsolova and Atanassov, 1994). Emershad and Ramming (1984) reported that multiple embryos might originate from somatic or zygotic cells of the ovule and the use of genetic markers might help to determine the origin of these embryos.

Cultured ovules were greenish to brown with or without callus at micropylar end. Ovules developed with or without endosperm and globular to torpedo shape embryos were recovered (Fig. 5.2.1E). Embryos were recovered from micropylar end of the ovule with cotyledons directed inside. Embryos were bright white and shiny. Cultured ovules processed an inner integument around the endosperm or embryo and a multi-layered outer integument. Abortive ovules were similar to non-abortive ovules with respect to integument. Mostly, single embryo (Fig. 5.2.1C) was observed in dissected ovules with the exception of two embryos in very few cases (Fig. 5.2.1D).

In the present study, direct somatic embryogenesis (proembryos and globular stage embryos) was observed along the hypocotyl, radicle junction of the embryos cultured in WPM supplemented with BA (0.89 µM) (Fig. 5.2.1G,H). Germinated embryos developed into plantlets which could be established on transfer to plastic cups containing a mixture of peat-sand-soil (1:1:1) (Fig. 5.2.1I).

5.2.3.3. Secondary somatic embryogenesis, germination of embryos and establishment of plantlets

Somatic embryos of Crimson Seedless induced from leaf, petiole, internodes, tendril and zygotic embryos induced further somatic embryos on hormones free half strength MS basal medium. Clusters of somatic embryos were observed via callus phase from primary somatic embryos (Fig. 5.2.2 A). Secondary embryos developed at the

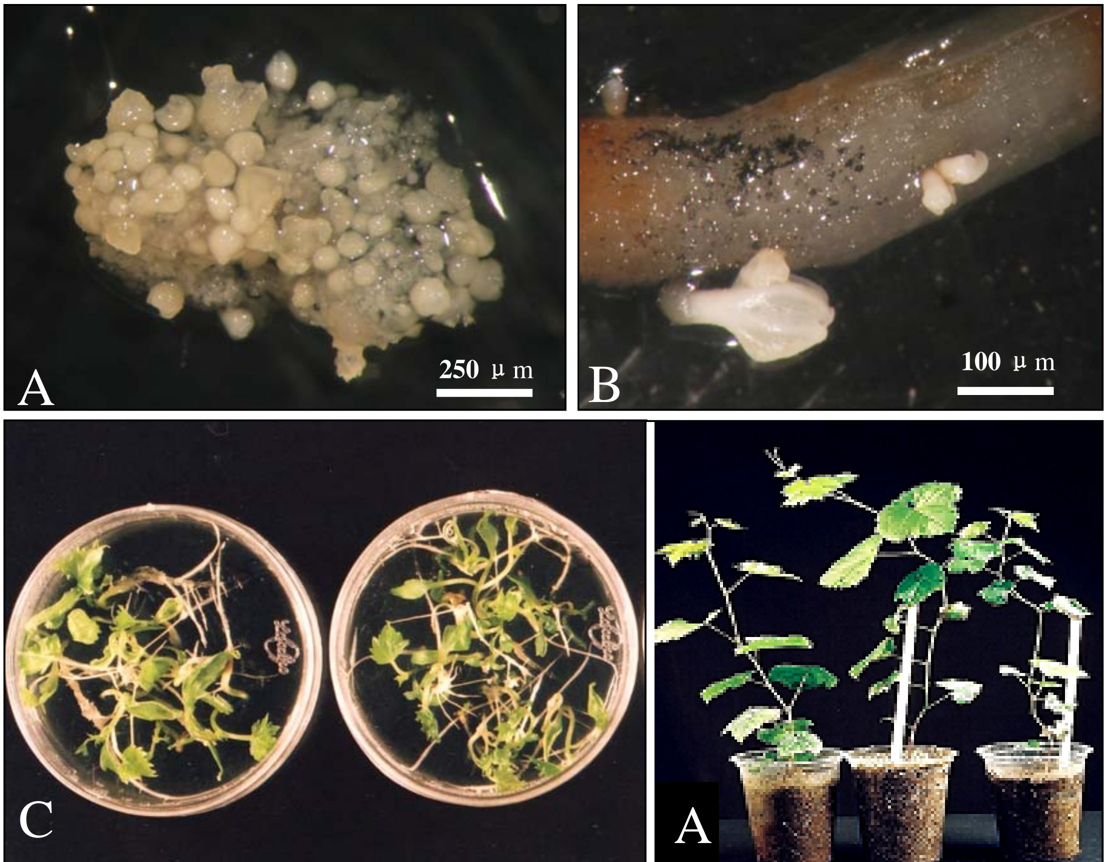


Fig. 5.2.2. Proliferation of somatic embryos (A), secondary embryos on hypocotyl (B), germinated embryos (C) and hardened plants (D).

radicle; hypocotyl junction of germinated embryos with or without intermittent callus as previously reported (Martinelli *et al.,* 1993). Occasionally embryos induced directly from hypocotyls (Fig. 5.2.2 B). Well formed mature somatic embryos germinated on different basal media containing BA and IBA at varied concentrations (Table 5.2.4). Maximum germination (60%) was obtained on WPM with or without BA (4.44 µM) and IBA (0.49 µM) (Fig. 5.2.2 C). Low germination efficiency of somatic embryos is common in grapevines and has been attributed to dormancy and embryo teratology (Mauro *et al.,* 1986; Gray *et al.,* 1989). Germinated embryos could be established on transfer to plastic cups containing a mixture of peat-sand-soil (1:1:1) (Fig. 5.1.2. D).

Somatic embryos could be distinguished from adventitious shoots by their bipolarity (shoot and root pole), and did not have any vascular connections with the underlying parental tissue as reported by Haccius (1978). Histology of mature somatic embryo confirmed the presence of bipolar vascular connections (Fig. 5.2.3A). Scanning Electron Microscopy observation revealed the induction of secondary somatic embryos (Fig. 5.2.3B).

Table 5.2.4: Influence of basal media and growth regulators on germination of somatic embryos of Crimson Seedless

Culture medium (µM)	No. of embryos inoculated	Germination %
NN	80	36.3
NN+BA (4.44)+ IBA (0.49)	100	29.0
NN+BA(0.44)+IBA (0.049)	80	35.3
WPM	70	60.0
WPM+BA(4.44)+IBA(0.49)	90	60.0
WPM+BA(0.44)+IBA(0.049)	55	46.4
½MS	90	44.4
½MS+BA(4.44) + IBA (0.49)	100	25.0
½MS+BA(0.44)+IBA (0.049)	40	44.4
SEM±	3.7	2.5
CD (p=0.01)	10.9	6.2
	**	**

** Significant at 1% level

We observed morphological abnormalities in secondary somatic embryos of Crimson Seedless (Fig. 5.2.4). Different morphotypes like embroys with one, two to multi-cotyledons, cabbage type, with long hypocotyl without radicle, with bulged hypocotyl, with slander cotyledons, fused embryos were recorded. Such aberrations in

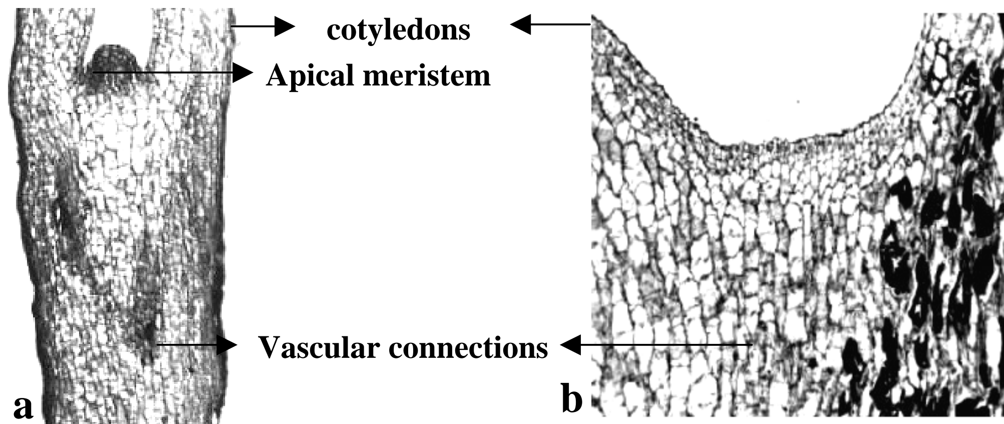


Fig. 5.2.3A. Histology of mature somatic embryos of Crimson Seedless with prominent apical meristem (a) and embryo lacking apical meristem (b).

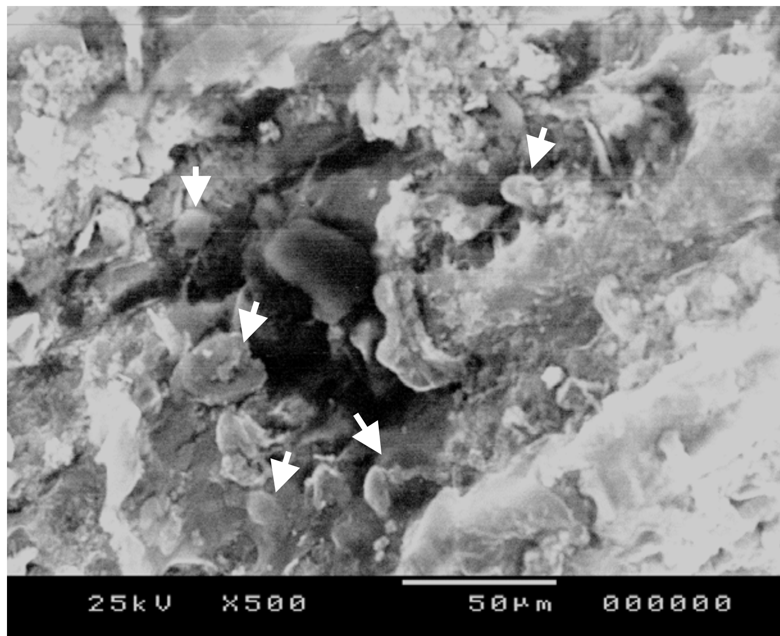


Fig. 5.2.3B. SEM view of mature somatic embryo showing secondary embryos

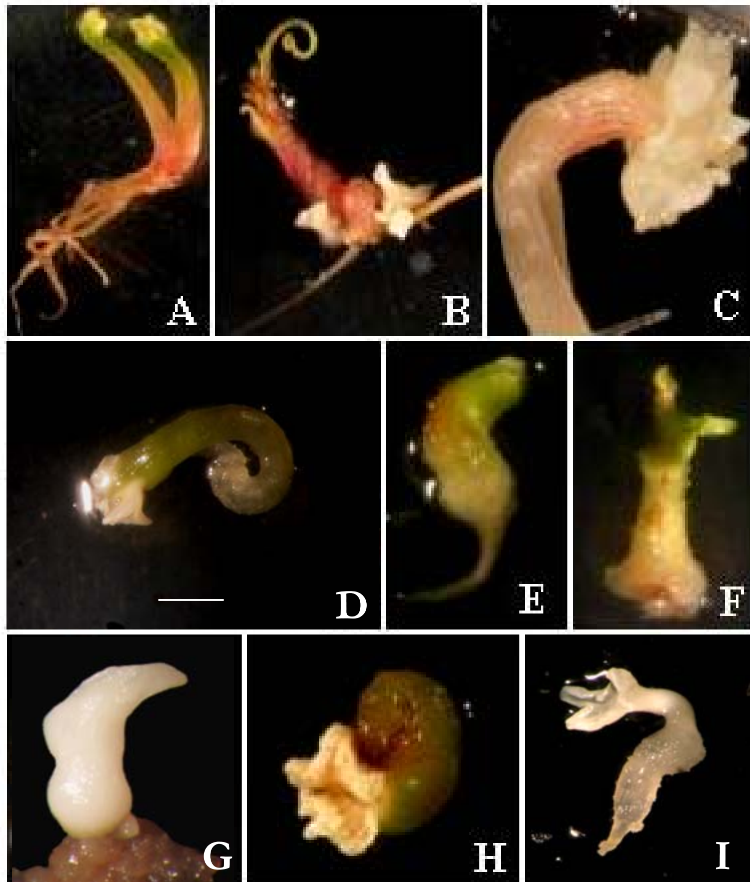


Fig. 5.2.4. Morphotypes of somatic embryos of Crimson Seedless. A-fused, B-twisted, C-cabbage type, D-without root, E-bulged hypocotyl, F-bulged hypocotyl without root, G- embryo with one cotyledon, H-bulged hypocotyl with two cotyledons and I-multicotyledonary embryo. Bar = 250 μm

somatic embryos of grapevines have been recorded earlier (Gray, 2000; Jayasankar *et al.* 2002) and are reported to be caused due to the influence of culture conditions, nutrient composition and growth regulators (Gray and Purohit, 1991; Gray, 2000).

In the present study, occurrence of abnormal somatic embryos may not be caused by exogenously supplied growth regulators, since secondary embryos induced in a medium devoid of growth regulators. Monocotyledonary embryos had normal to superior growth and development compared to dicotyledonary embryos. This could be a result of larger shoot apical meristematic region compared to dicotyledonous somatic embryos. Between the monocotyledonary and dicotyledonary embryos, conversion to plantlets and subsequent establishment did not differ significantly (data not shown), suggesting that, monocotyledonous morphotypes are capable of functioning like normal somatic embryos.

5.2.4. Conclusion

The use of *in-ovulo* embryo rescue technique has application in production of hybrid progenies having both seeded and seedless parents, which could facilitate genetic studies leading to an understanding of the inheritance of seedlessness trait. The ovule culture technique in seedless cultivars can lead to a higher number of progeny thus increasing the efficiency of breeding programs. The strategy of *in-ovulo* embryo rescue method for induction of somatic embryogenesis from immature zygotic embryos could also be a support to the germplasm enhancement programmes. Success of raising plants from weak and immature embryos largely depends on their stage of maturity and composition of the culture medium as observed in the present study. Development of an *in vitro* technique to culture normally abortive ovules from seedless grapes would permit hybridization among seedless cultivars.

Part of the work has been reported in the following publications:

Nookaraju, A., Barreto, M.S., Karibasappa, G.S. and Agrawal, D.C. (2007) **Synergisitic effect of CPPU and benzyladenine on embryo rescue in six stenospermocarpic cultivars of grapevine**. *Vitis* 46(4): 188-191.

5.3. Influence of polyamines on maturation and germination of somatic embryos

5.3.1. Introduction

Growth and development of somatic cells of higher organisms is regulated by multiple controls. Somatic embryos of grapevine multiply repeatedly and often fail to mature and germinate due to factors like dormancy and embryo teratology. Apart from plant growth regulators, polyamines (PAs) have been reported to play a significant role in maturation and germination of somatic embryos in eggplant (Fobert and Webb, 1988) and carrot (Mengoli *et al.,* 1989). Many plant processes regulated by different phytohormones have been correlated with PA metabolism (Kaur-Sawhney *et al.,* 2003). PAs, spermidine (SPD), spermine (SPM) and their diamine obligate precursor putrescine (PUT), are small aliphatic amines that are ubiquitous in all plant cells. Though the precise role of PAs is yet to be understood, extensive studies suggest their role in variety of physiological processes ranging from cell growth and differentiation to stress responses.

PAs, like PUT behave like cations at their physiological pH and can interact with anionic macromolecules like DNA, RNA, acid phospholipids and proteins (Schuber, 1989) and modify different plant processes. PAs have also been implicated in a wide range of biological processes, including growth, development and abiotic stress responses (Minocha *et al.* 1995). PAs have been reported to be key regulatory elements in morphogenesis during somatic and zygotic embryo development in grapevine (Faure *et al.,* 1991). The cellular accumulation of PAs in relation to different plant morphogenic processes has not been studied extensively. Earlier reports have indicated correlation between cellular PA levels and maturation and germination of somatic embryos of several plant species (Faure *et al.,* 1991; Yadav and Rajam, 1997; Minocha *et al.,* 1999). However, correlation between PAs and their biosynthetic enzymes and different plant growth processes are not universal and may be species dependant (Evans and Malberg, 1989). Though PAs were known to influence cell differentiation leading to somatic embryogenesis (Feirer *et al.,* 1984), their time and duration dependant effects and the precise role of PAs in the regulation of somatic embryogenesis still remains unclear. PAs have been reported to enhance regeneration of roots, shoots and embryos, delay or prevent senescence, and regulate flowering in various plant species.

In our laboratory, we could establish pro-embryonal masses (PEM) of grapevine cv. Crimson Seedless from different explants, however, the frequencies of embryo maturation and germination were low resulting in poor embryo conversion. The PEM

consisted of embryogenic calli with small profuse globular pro-embryoids. The present study was carried out to study the correlation of PAs with maturation and germination of somatic embryos from PEM of Crimson Seedless.

5.3.2. Materials and methods

5.3.2.1. Influence of different PAs on maturity and germination

Pro-embryonal mass (PEM) of Crimson Seedless obtained from somatic embryo of the cultivar were cultured on half strength MS basal medium for further proliferation. To improve the percentage of maturation and germination, PEM were inoculated on half strength MS basal medium containing BA (0.89 μM) and PAs - putrescine (PUT) (0.1-100 μM), spermine (SPM) (0.4-40 μM), spermidine (SPD) (1-200 μM). These concentrations of PAs were optimized in a separate experiment (data not shown). Sucrose (3%), agar (0.65%) and charcoal (0.2%) were added to all media. Five PEM (500 mg each) were inoculated per petridish (55 mm) and each treatment consisted of twenty-five replicates. Experiment was repeated thrice. The cultures were incubated at 16 h photoperiod with a light intensity of 12.2 μmhos $cm^{-2}s^{-1}$ at 25 ± 2°C. Observations on maturation (showing well formed globular to torpedo shape embryos) and germination were recorded at weekly interval and the data were subjected to ANOVA.

5.3.2.2. Extraction of Polyamines

For HPLC analysis of PAs, PEM (200 mg) under each treatment was ground in 2 ml of 4% perchloric acid and homogenized. The mixture was kept at 4°C for 1 h. The samples were then mixed gently for 1 min and kept again on ice for 4 min. Then the samples were centrifuged for 5 min at 5000g and filtered through glass wool. Supernatant was transferred to a glass vial for benzoylation.

5.3.2.3. Sample preparation for HPLC analysis

The PA standards and samples were benzoylated according to the method described by Flores and Galston (1982) with minor modifications.

1. One ml of 2N NaOH and 5 μl of benzoyl chloride added to 200 μL of HClO4 extract in glass vial and the contents were vortexed for 30 s.
2. After incubation of the above mixture at 25°C for 20 min, saturated NaCl (2 ml) was added to the mixture to stop the reaction.
3. Then 3 mL of diethyl ether was added to the mixture and the contents were mixed thoroughly.

4. The samples were centrifuged at 1500 g for 5 min and 1.5 ml of upper ether phase was collected and evaporated over a water bath (60°C).
5. The benzoylated PAs were re-dissolved in 100 µl of 64% (v/v) methanol (HPLC grade; Merck, Germany) for HPLC analysis.
6. The benzoylated samples were stored at –20°C until used for HPLC analysis.

Benzoylated PAs were analyzed with the Waters 2690 separation module HPLC equipped with 2487 Dual absorbance detector (Waters, USA). A delta pack C-18 column (4.6 X 250 mm, 15 µm particle size; Waters, USA) was used for the separation of PAs. The benzoylated PA (50 µl) were injected manually and chromatographed at 28°C. The solvent system consisted of methanol:water. Samples were run isocratically at 60% methanol (v/v), with a flow rate of 0.5 ml min^{-1}. The benzoylated PAs were detected spectrophotometrically at 254 nm. The regression curves of each PA sample allowed quantitative estimation of PA in the sample. Amount of PA in the sample was expressed as µmol g^{-1} FW.

5.3.3. Results and Discussion

5.3.3.1. Influence of polyamines on maturation and germination of somatic embryos from PEM of Crimson Seedless

Percentage of maturation and germination of embryos from PEM significantly depended on type of PA in the medium and incubation period. Among the three PAs tested, PUT affected the maximum maturation and germination. At 30 d of incubation, PUT resulted in 92.2% of maturation and 84.6% of germination (Table 5.3.1) (Fig. 5.3.1). Between SPD and SPM, the later affected higher percentage of maturation and germination in the cultivar. In all the treatments, except PUT, the maximum maturation and germination was affected at 30 d of incubation. In the medium supplemented with PUT, maximum maturation and germination was observed at 30 and 21d, respectively. PEM without PA treatment (Control) though resulted in high maturation % (82.1 at 30 d), however, germination percentage was lower (31.6 at 30 d), hence was the need to carry out the present investigation. On comparison of the results of media supplemented with SPD or SPM with control, it was observed that at 30 d, both the PAs did not improve the maturation efficiency, though SPM resulted in higher germination percentages (Table 5.3.1).

The HPLC profiles of standard PA showed that absorption peaks for PUT, SPD and SPM were observed at 8.2, 10.75 and 15.2 min, respectively (Fig. 5.3.2). The highly

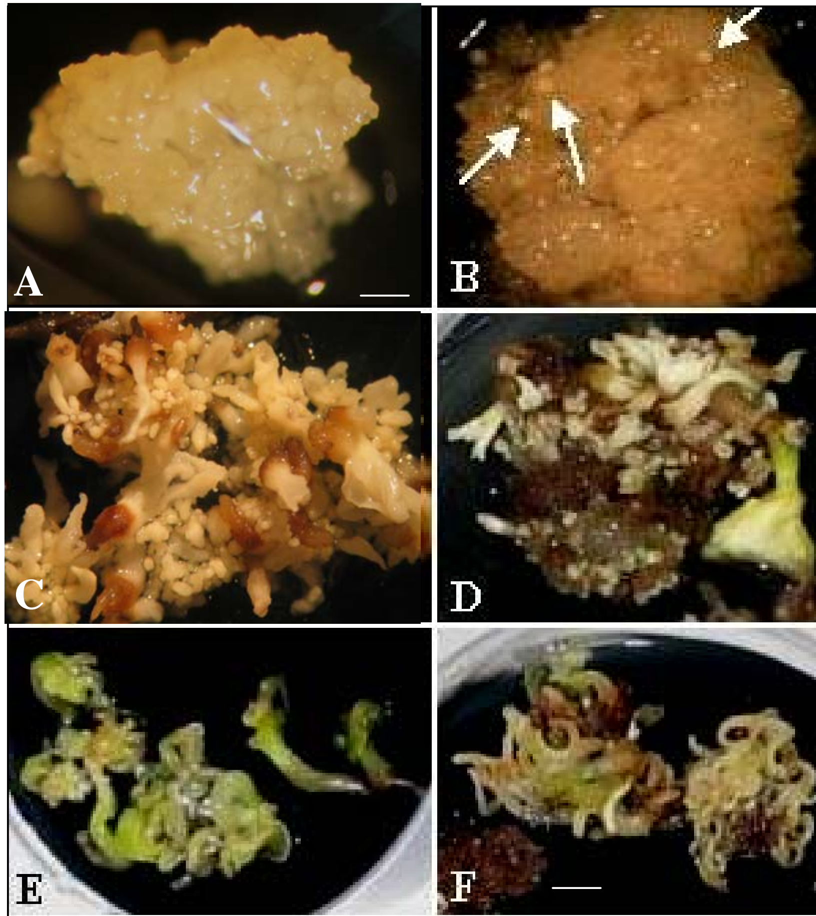


Fig. 5.3.1. Stages of PEM maturation and germination. A: PEM, B: PEM showing globular somatic embryos, C&D: PEM showing matured embryos and E&F: PEM with germinated embryos. Bar = 100 µm

reproducible retention times obtained with HPLC of PA standard allowed for preliminary identification of the unknown peaks in PEM tissue extracts.

In the present study, only free cellular PAs were estimated by HPLC, as these were considered to be abundant and the only active forms (Bagni *et al.,*1994). HPLC analysis showed that the accumulation of cellular PUT in PEM was the maximum at 14 d in the medium supplemented with PUT (10 μM). At 21 and 30 d, cellular PUT levels decreased gradually (Table 5.3.1). A similar decreasing trend in cellular PUT levels from 14 d onwards was observed when medium was supplemented with SPD or SPM (Fig. 5.3.3A). In control (medium without PA), cellular PUT levels were lower compared to PA treatments and their levels gradually increased towards 30 d after inoculation, where the frequencies of maturation and germination of somatic embryos from PEM of the cultivar were highest. From the study, it was observed that the quantity of cellular PUT level at the start of inoculation (0 d) was lower (1.1 μmol g^{-1} FW) and increased gradually reaching the limit (6.8 μmol g^{-1} FW) at 14 d (Table 5.3.1). Increase in PUT content corresponded to a drastic rise in percentage of maturation and germination of somatic embryos from the PEM. In conformity with our findings, Helior *et al*. (1998) also observed higher PUT content in the tissues with the advancement of maturity. However, in contrast to PUT, cellular levels of SPD were higher in the beginning and declined gradually showing an inverse correlation with maturation and germination (Table 5.3.1) (Fig. 5.3.3B).

These results demonstrate that PUT is one of the most predominant PA supporting maturation and germination of embryos in PEM of Crimson Seedless. Higher titers of PUT were measured during somatic embryogenesis in *Daucus carota* (Mengoli *et al*. 1989), while SPD was most abundant during development of somatic embryos of *Pinus radiata* (Minocha *et al*. 1999) indicating that the requirement of PA during somatic embryogenesis may be species dependant.

The PUT/SPD ratio has followed the similar trend as that of PUT content and showed a positive correlation with maturation and germination of embryos from PEM. The PUT/SPD ratio was highest (4.0) in PEM cultured on medium supplemented with PUT at 14 d of inoculation. The ratio of PUT/ SPD+SPM was also maximum (2.5) in the medium supplemented with PUT (10 μM) at 30 d after inoculation. Higher values of the PUT/SPD ratio were attributed to the higher levels of PUT and lower levels of SPD in the PEM as reported earlier (Yadav and Rajam 1998). Ratios of PUT/SPD increased from 0.2 at 0 d to 4.0 at 14 d after inoculation while PUT/SPD+SPM ratio increased gradually from 0.1 at 0 d to 2.5 at 30 d (Table 5.3.1) in the medium supplemented with PUT. It has

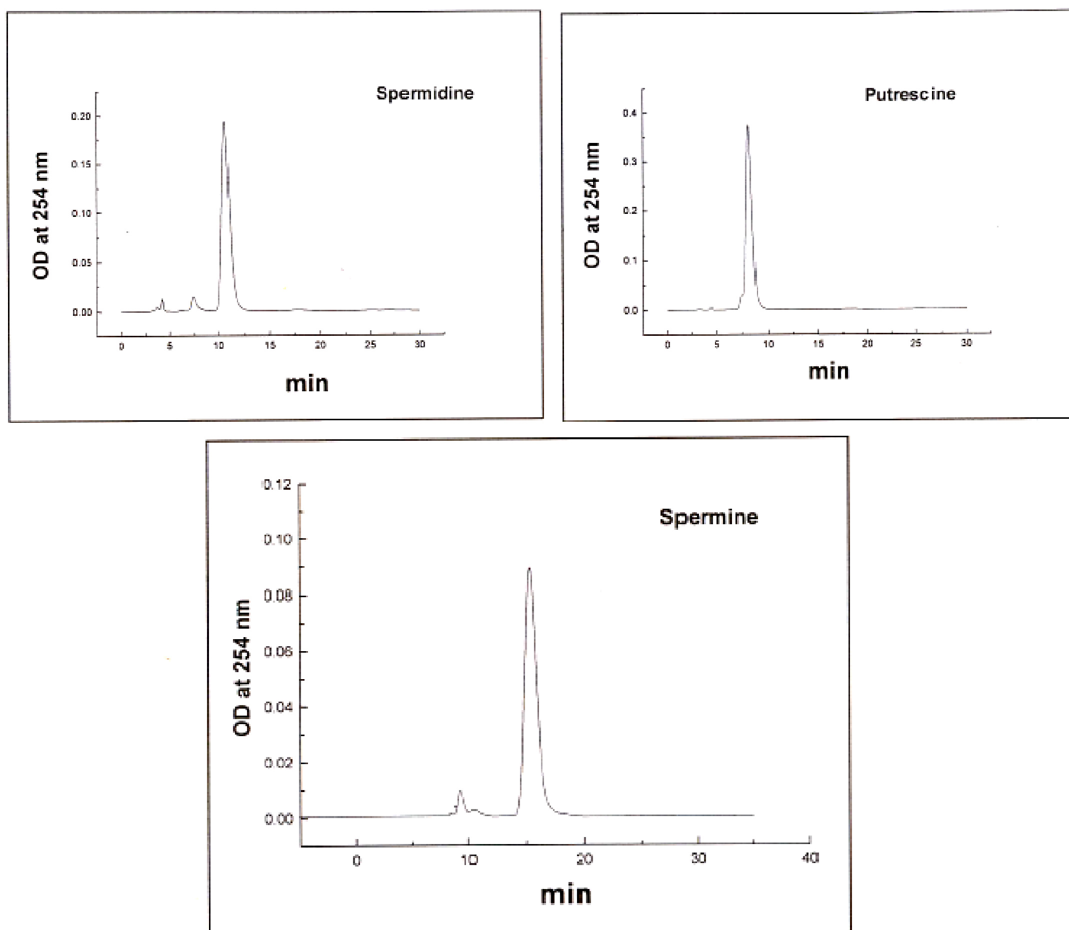


Fig. 5.3.2. Retention peaks of standard PAs

been documented earlier that PUT/SPD+SPM ratio was higher in mature tissues than the juvenile ones (Helior *et al*. 1998). Cellular PA levels and their PUT/SPD ratio have been suggested as the important determinants of plant regeneration ability in *indica* rice (Shoeb *et al*. 2001).

Table 5.3.1: Cellular PA contents in PEM of Crimson Seedless cultured in the media supplemented with different PAs

Treatment	Days after inoculation	Maturation %	Germination %	PUT (µmol g^{-1} FW)	SPD (µmol g^{-1} FW)	PUT/SPD	PUT / SPD+SPM
Initial Explant	0	-	-	1.1	6.6	0.2	0.1
PUT (10 µM)	7	69.3	8.9	3.6	2.7	1.3	0.1
	14	86.6	74.7	6.8	1.7	4.0	0.1
	21	90.0	84.6	5.9	2.2	2.7	1.0
	30	92.2	84.6	4.9	1.9	2.5	2.5
SPD (50 µM)	7	00.0	00.0	3.3	8.0	0.4	0.3
	14	56.2	12.3	4.7	4.9	1.0	0.1
	21	74.7	24.5	4.0	3.9	1.0	0.9
	30	86.2	30.6	2.1	2.0	1.0	1.0
SPM (40 µM)	7	00.0	00.0	2.3	2.0	1.2	0.4
	14	67.2	26.3	3.9	1.7	2.4	0.1
	21	82.7	49.2	2.8	1.9	1.5	0.8
	30	85.1	55.8	1.8	1.8	1.0	1.0
Control	7	00.0	00.0	2.4	1.6	1.5	1.5
	14	14.7	00.0	3.1	10.3	0.3	0.2
	21	56.3	12.3	3.5	3.7	0.9	0.9
	30	72.3	42.6	4.3	1.9	2.3	2.0
SEM±		1.6	2.9	0.3	0.5	0.4	0.3
CD (p=0.01)		6.1	11.3	0.9	1.4	1.2	1.2
		**	**	**	**	**	**

*Basal medium – half strength MS + BA (0.89 µM), ** Significant at 1% level

In addition to cellular PAs estimated from PEM during culture (at weekly interval), amount of residual PAs present in the media before (0 d) and during culture (at weekly interval) was estimated. Initial PUT level (0 d) in media supplemented with PUT (10 µM) was 8.8 µmol g^{-1}. This level decreased gradually in culture media (Table 5.3.2). In case of medium supplemented with SPD, SPD level in medium decreased from initial level of 6.2 µmol g^{-1} to 0.4 µmol g^{-1} at the end of the culture period. Similar trend was observed for SPM also. The decrease in residual levels of PUT and SPM in their respective media was sharp as compared to residual SPD in media supplemented with SPD (Fig. 5.3.4). In all the media supplemented with any one of the PA, all the three PAs were detected at any point of time indicating inter-conversion of different PAs in the media during culture.

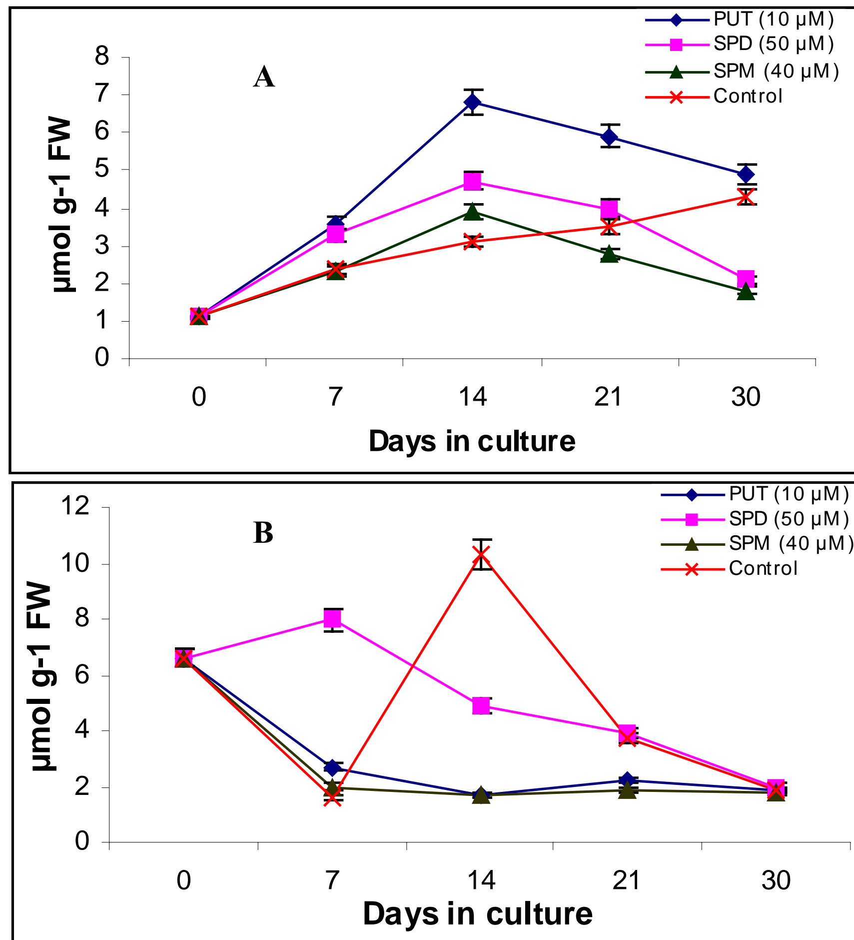


Fig. 5.3.3. Trends in cellualr PAs in the PEM of Crimson Seedless. A: Cellular PUT levels in PEM cultured in the medium supplemented different PAs and B: Cellular SPD levels in PEM cultured in the medium supplemented different PAs. Bars represent ±SE of three replicates.

Table 5.3.2: PA contents in the media supplemented with different PAs

Medium composition	Days of incubation	PUT (µmol g^{-1})	SPD (µmol g^{-1})	SPM (µmol g^{-1})
PUT (10 µM)	0	8.8	-	-
	7	3.7	0.7	2.6
	14	2.7	0.7	11.6
	21	1.2	0.9	9.3
	30	0.7	0.8	0.0
SPD (50 µM)	0	1.6	6.2	0.9
	7	1.6	5.8	13.2
	14	1.5	3.1	6.1
	21	1.6	0.9	2.0
	30	1.9	0.4	0.0
SPM (40 µM)	0	5.1	0.5	86.4
	7	1.5	1.3	39.6
	14	1.6	3.2	21.4
	21	1.0	4.1	2.1
	30	1.2	1.2	0.0
SEM		0.7	0.4	3.3
CD (p=0.01)		2.6	1.5	13.0
		**	**	**

*Basal medium – half strength MS + BA (0.89 µM), ** Significant at 1% level

Previous reports on PAs indicate their role in somatic embryo development (Minocha *et al.,* 1995). Changes in cellular PA metabolism during somatic embryogenesis have been reported earlier for several plant species (Minocha *et al.* 1995; Kumar *et al.* 1997). Higher levels of PAs have been associated with somatic embryogenesis in carrot (Fienberg *et al.* 1984). In the present study, higher cellular levels of PUT in the beginning might have stimulated maturation of PEM as reported earlier (Andersen *et al*. 1998). Increased activities of arginine decarboxylase (ADC) and S-adenosylmethionine decarboxylase (SAMDC) have been reported as a result of higher PUT levels in tissues (Fienberg *et al*. 1984; Yadav and Rajam 1997). It was found that cellular SPD levels were least at 14 d in the cultivar. An inverse correlation was observed between cellular PUT and SPD levels in the PEM of the cultivar cultured either in PUT or SPD indicating an inter conversion of PUT and SPD by the action of enzymes in the PEM (Tassoni *et al.,*2000). A gradual decrease in PUT level in PEM with the advancement of culture period could be due to utilization of PAs during maturation and germination as reported earlier (Yadav and Rajam, 1997). In another study on grapevine, increase in PUT content had correlation with the maturation response in Pinot noir (Helior *et al.* 1998). Decrease in the PA levels was observed during embryo transition from globular stage to

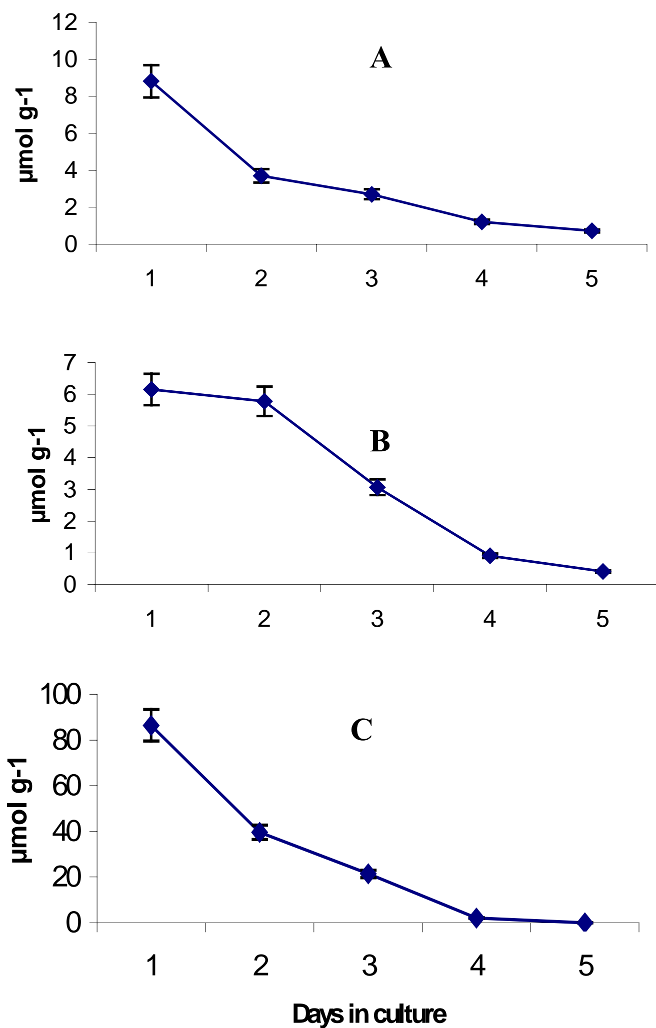


Fig. 5.3.4. Levels of residual PAs in their respective media. A: Residual PUT in the medium supplemented with PUT (10 µM). B: Residual SPD in the medium supplemented with SPD (50 µM). C: Residual SPM in the medium supplemented with SPM (40 µM). The values are the mean±SE of three replicate samples.

development of plantlets (Bertoldi *et al.* 2004). In our study, PUT levels were higher during maturation stage, while its level decreased during germination. These results are in conformity with earlier reports (Martinelli *et al.,* 2001; Bertoldi *et al.,* 2004), where a gradual decrease in PUT content was found to have correlation with high efficiencies of embryo germination and conversion to plantlets.

In general, cells undergoing expansion and elongation contain low levels of free PAs synthesized via arginine decarboxylase (ADC) (Galston and Kaur-Sawhney, 1995). Cellular PUT levels increased substantially with addition of PAs to the media indicating a rapid uptake by PEM cells. PUT levels had a positive correlation with maturation, hile, a reverse trend was observed with cellular SPD levels in PEM. A gradual decrease in cellular SPD content from bullet shape embryos stage to cotyledonary stage of somatic embryos of *Pinus radiata* has been reported (Minocha *et al.,* 1999). It was earlier observed in *Arabidopsis* that SPD supplied exogenously could largely be taken up from the culture medium and rapidly translocated to cotyledons (Tassoni *et al.,* 2000). The presence of SPD and SPM in the media supplemented with PUT at later stages of culture could be attributed to the synthesis of SPD and SPM from their immediate precursor, PUT in the PEM tissues. The rate of uptake of PAs from the medium by PEM had a strong correlation with the frequency and earliness in maturation and germination of somatic embryos of PEM of grapevine cultivar Crimson Seedless.

5.3.4. Conclusion

Present study on PAs demonstrates the usefulness of exogenous supply of Putricine (PUT) in affecting maturation and germination of somatic embryos from pro-embronal masses (PEM) of grapevine cultivar Crimson Seedless. Cellular polyamines (PAs) levels in PEM had correlation with morphogenetic changes. The residual PAs measured in the media showed depletion with culture period indicating its ready uptake by PEM, and thereby affecting maturation and germination of somatic embryos.

Part of the work has been reported in the following publications:

Nookaraju, A., Barreto, M.S. and Agrawal, D.C. (2008) **Cellular polyamines and maturation and germination of somatic embryos from pro-embryonal masses of two grapevine cultivars.** *Vitis* 47(1): (In press).