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Rapid *in vitro* propagation of grapevine cv. Crimson Seedless— Influence of basal media and plant growth regulators

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Abstract

Grapevine genotypes differ in tissue culture requirements and thus require optimized culture conditions for *in vitro* propagation. Single node segments of Crimson Seedless cultured on six different basal media *i.e.* Murashige and Skoog (MS), Eriksson (ER), Gamborg (B5), Nitsch and Nitsch (NN), Woody plant medium (WPM) and Chee and Pool (C_2d) showed different percentage of shoot initiation and morphogenetic responses. The maximum shoot initiation (90.0%) was observed in MS medium. Except ER, all other media induced rooting at the base of nodal segments in varying percentages though number and quality of roots and their establishment on transfer to pots varied greatly. WPM induced the maximum rooting in nodal segments (69.1%) with establishment rate of 100.0%. Induction of multiple shoots in nodal segments was achieved on inclusion of 6-benzyl adenine (BA) (8.87 µM) and indole-3-butyric acid (IBA) (1.48 µM) in the MS medium. In second sub-culture *i.e.,* at 90 days, shoot bud proliferation could be increased many fold on transfer of these initial shoot clumps to glass bottles instead of culture tubes. The maximum average number of primary shoots (19.5 per explant) was achieved on MS with BA (8.87 µM) and IBA (1.48 µM). Elongation of shoots was achieved on MS with BA (2.22 µM) + α-naphthalene acetic acid (NAA) (0.54 µM). Induction of *ex vitro* rooting and establishment of rooted shoots after transfer to pots was achieved in different efficiencies when shoots were given pulse treatment of indole-3-acetic acid (IAA) or IBA or NAA at 57.08, 49.0 and 53.71 µM, respectively, for 5 or 10 min. Survival of *in vitro* and *ex vitro*-rooted shoots on potting was 90.0 and 100.0%, respectively.

Key words: Crimson Seedless, grapevine, micropropagation, *Vitis.*

Introduction

In grapes, response of different cultivars to *in vitro* multiplication varies (Barlass and Skene, 1980; Botti *et al.*, 1993). Hence, this necessitates the optimization of micropropagation procedure for different cultivars, clones or newly introduced varieties. Shoot apex as an explant was commonly used for micropropagation of herbaceous species (Murashige, 1977; Abbot, 1978) but to a lesser degree in woody species. Grapevine was among the first woody plants, where the use of shoot apices and axillary buds for *in vitro* propagation of various species and cultivars of *Vitis* was reported. *In vitro* propagation of vines could be obtained by culture of shoot apices (Harris and Stevenson, 1979; Goussard, 1981) and adventitious shoot formation from fragmented apices (Barlass and Skene, 1978). Use of other explants like meristem in *Vitis rotundifolia* (Thies and Graves, 1992), microcutting and axillary buds in *Vitis* x *Muscadania* hybrids (Torregrosa and Bouquet, 1995) have also been documented. Despite a moderate multiplication rate, nodal segment remains a widely used explant in micropropagation of vines due to its operational feasibility and genotype stability (Torregrosa *et al.*, 2001). *In vitro* propagation could be obtained by axillary shoot initiation in nodal cuttings (Galzy, 1969). Mhatre *et al.* (2000) used nodal segments as explants to propagate three *vinifera* varieties. Studies on *in vitro* culture and propagation of vines have recently been reviewed by Torregrosa *et al.* (2001).

Crimson Seedless, a red table grape variety was developed by Ramming and Tarailo of the USDA, Fresno, California, USA as a result of cross between Emperor and C33-199 (Dokoozlian *et al.*, 1998). Retail trade over there has received the variety favorably due to its excellent eating characteristics like crisp and firm berries. *In vitro* propagation offers an advantage of clonal multiplication of desired material at faster rate and on a continuous basis. To the best of our knowledge, there is no report available for *in vitro* propagation of Crimson Seedless. The presernt paper deals with the Crimson Seedless specific micro propagation requirements.

Materials and methods

Plant material: Twigs of field grown vines of Crimson Seedless were collected from the vineyard of National Research Centre for Grapes, Manjri, Pune, India. Single node segments (1.5-2 cm long) were used as explant for culture. Nodal segments were surface sterilized by soaking them in liquid soap solution for 10 min followed by thorough rinses with running tap water. The explants were then submerged in 0.1% fungicide solution (Bavistin™, BASF, India) for one hr followed by 2-3 washes with sterile distilled water. Then the explants were treated with 0.1% (w/v) mercuric chloride for 10 min followed by several rinses with sterile distilled water in a laminar flow hood. Excess water was removed by blotting dry the explants on a sterile filter paper.

Shoot initiation: For shoot initiation, nodal segments were inoculated in glass culture tubes containing following six different basal media: MS (Murashige and Skoog, 1962), ER (Eriksson, 1965), B5 (Gamborg *et al.*, 1968), NN (Nitsch and Nitsch, 1969), WPM (Llyod and McCown, 1981) and C_2d (Chee and Pool, 1987). Different workers have reported *in vitro* propagation of grapevine

employing various basal media. Based on the earlier reports, these six media were selected to determine the optimum basal medium for shoot initiation and other morphogenetic processes for the cultivar Crimson Seedless.

To maximize the shoot initiation response, another experiment was set with MS basal medium supplemented with BA (0.44 µM-44.38 µM). Each culture tube had only single nodal segment.

Induction of multiple shoots: For induction of multiple shoots, primary nodal segments from field grown vines as well as secondary nodal segments excised from *in vitro* grown shoots from primary nodal segments were inoculated on MS basal medium supplemented with different growth regulators like BA (8.87 µM) alone or in combination with IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM). Explants with induced multiple shoots were shifted to fresh media after every 4 weeks.

Elongation of multiple shoots: For elongation, shoots clumps were kept on MS basal medium supplemented with BA (2.22 µM) alone or in combination with IAA (0.57-1.71 µM) or IBA (0.49 -1.48 µM) or NAA (0.54-1.61 µM).

***In vitro* rooting of shoots**: Elongated shoots were transferred to culture tubes containing half strength semi-solid or liquid MS basal medium supplemented with IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM). Agar (0.65%) or gelrite (0.2%) were used as gelling agents.

Sucrose (2%) as a carbon source was added to all the media and pH adjusted to 5.8 before autoclaving at 121^{0}C and 105 KPa for 20 min. All the growth regulators were added before autoclaving.

***Ex vitro* rooting**: Three auxins, IAA, IBA and NAA at a concentration of 57.08, 49.0 and 53.71 µM, respectively, were used for pulse treatment. Elongated shoots (4.5-5.0 cm) were given pulse treatment for 5 or 10 min. After pulse treatment, shoots were transferred to plastic cups consisting of a mixture of sterile peat: soil: vermiculite (1:1:1). Plants were irrigated with ¼ strength of MS salts medium without sucrose and covered with thin and transparent polythene sachets and placed in growth room with 24h light with an intensity of 24.4 µ mol m^{-2} s^{-1} at 25 ± 2^{0}C. Untreated shoots, which served as control, were also transferred to the same potting mixture and growth conditions.

Hardening of plants: Shoots rooted under *in vitro* conditions and nodal segments with direct rooting in basal media were transferred to plastic cups containing a mixture of soil and sand (1:1). Sachet technique followed by Ravindra and Thomas (1995) and Bharathy *et al.* (2003) was used for hardening of *in vitro* and *ex vitro*-rooted shoots and nodal segments with induced direct rooting in basal media. Plants were covered with thin, transparent polythene sachets and kept in growth room having 24h light with intensity of 24.4 µ mol m^{-2} s^{-1} at 25 ± 2^{0}C. After 2 weeks, plants were shifted to another room having ambient temperature. Here, sachets were cut at top corners and were removed completely after 2 weeks. After that plants were transferred to a polyhouse.

Observations of all the experiments were taken at 30 days interval. Data were analyzed using ANOVA (Spiegel, 1992).

Results and discussion

Shoot initiation: Shoot initiation in nodal segments commenced after 7 days of inoculation and continued up to 25 days. Out of six nutrient media tested, the maximum shoot initiation in explants was observed in MS basal medium (90.0%) followed by NN (89.4%) and WPM (87.2%) after 30 days of inoculation (Table 1). Percent of explants showing shoot induction was highest (85.7) in MS followed by 78.8 in NN and 78.7 in WPM.

Six different basal media had varying influence on induction of direct rooting in explants. With the exception of ER, all other basal media induced rooting at the basal end of nodal segments (Fig. 1A). The maximum response (69.1%) was observed in WPM followed by B5 (60.0%) though establishment of rooted nodal segments into plants on potting was 100.0 and 66.7%, respectively. Direct rooting in nodal segments has advantage in micropropagation, since explants rooted in this manner can directly be transferred to pots and hardened plantlets can be obtained after 2 months. Also use of single node cuttings in culture instead of 3-4 node cuttings used in vineyard can give larger number of plants if source of mother material is a serious limitation.

Induction of shoots in nodal segments could be increased to 100.0% on incorporation of BA at 4.44 µM in MS basal medium (data not shown). There was no rooting at the base of explants in any of the BA concentration unlike BA free MS basal medium. In a study with different grapevine cultivars and rootstocks, Roubelakis-Angelakis and Zivanovitc (1991) reported increased rhizogenic effect in single node segments on medium containing lower amounts of N, K, Ca and Mg. In our study, WPM contains

Table 1. Effect of different nutrient media on morphogenetic responses in Crimson Seedless

Nutrient Medium	Number of explants Inoculated	Explants showing shoot initiation (%)	Explants developed shoots (%)	Average length of shoots (cm) ± SD	Explants showing rooting at base (%)	Average number of roots per explant ± SD	Plants established on potting (%)
MS	70	90.0	85.7	1.36±0.26	25.0	3.67±0.26	33.3
ER	45	75.6	66.7	1.54±0.50	00.0	0.00±0.00	00.0
B5	69	60.9	53.7	1.75±1.00	60.0	6.03±0.67	66.7
NN	47	89.4	78.8	1.30±0.47	55.8	6.75±0.67	00.0
WPM	47	87.2	78.7	1.57±0.74	69.1	6.52±1.70	100.0
C_2d	46	84.8	78.3	1.36±0.16	22.0	2.11±0.08	25.0
LSD (*P*=0.05)		15.2	11.4	0.49	5.60	1.30	8.1
		*	**	**	**	*	**

* Significant at 5%; ** significant at 1%.

Explant: Primary single node segment. Observations recorded after 30 days of inoculation.

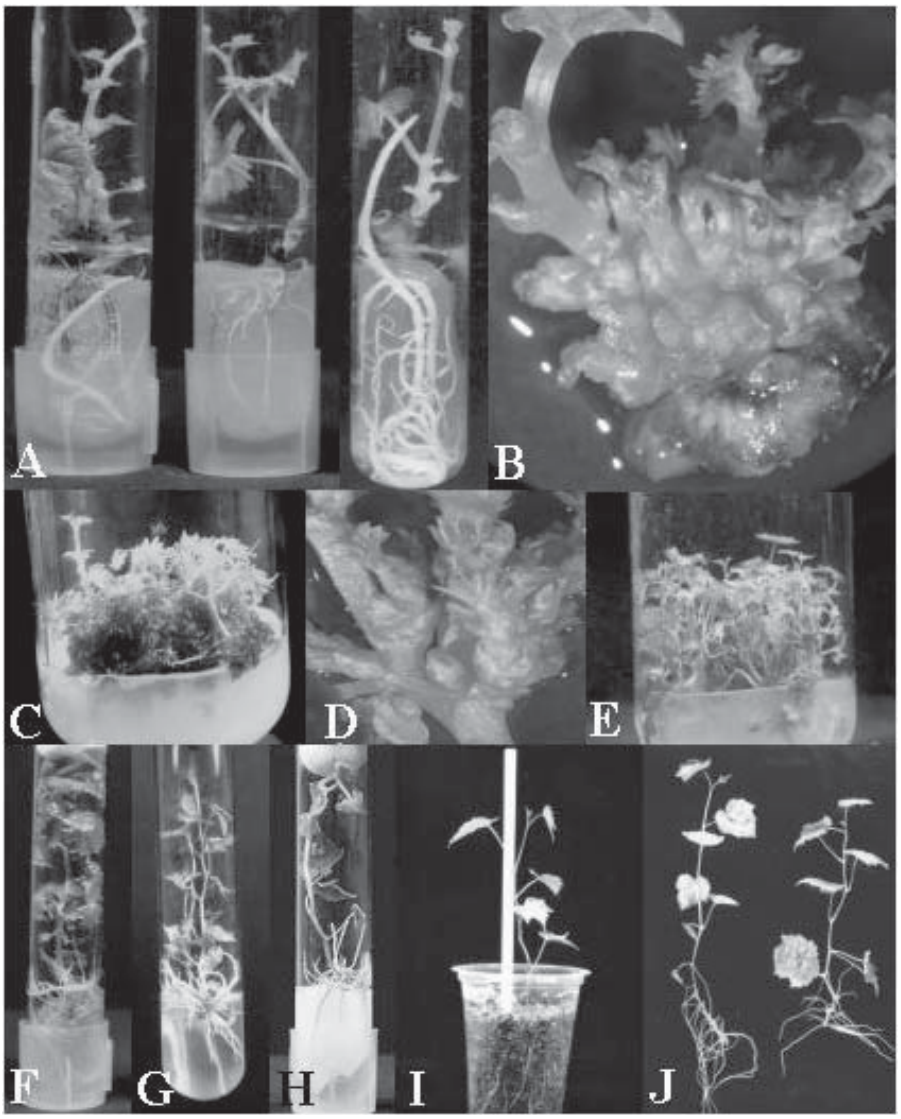

Fig. 1. Direct rooting in single node explants (A); Multiple shoots in secondary nodal segment (B); Proliferation of multiple shoots (C); Shoot buds in axils (D); Elongated shoots (E); Rooted shoot in medium with agar (F); Rooted shoot in medium with gelrite (G); Rooted shoot on filter paper bridge in liquid medium (H); *Ex vitro* rooted shoots (I & J).

lower amount of nitrogen compared to other five media tested and hence could be a reason for increased rooting response in nodal segments. Similarly, maximum amount of nitrogen present in ER medium compared to all other media tested could be a reason for absence of rooting in the ER medium.

Multiple shoot induction: In preliminary trials conducted to optimize the ideal concentration of BA for multiple shoot induction, BA concentration 8.87 µM was found to be better for inducing multiple shoots in higher percent of explants. In further experiments, various auxins were tried along with BA at this concentration to maximize multiple shoot induction. In two separate experiments; both primary as well as secondary nodal segments were used to induce multiple shoots. An average of 2.71 shoots per primary nodal segment could be induced on MS medium with BA (8.87 µM) and IBA (0.98 µM) in 37.0% of explants after 30 days of inoculation (data not shown). More or less similar observations were recorded for secondary nodal segments too (Table 2). Out of 3 auxins tested, IBA at 0.98 µM added to MS medium containing BA (8.87 µM) induced multiple shoots in 25.0% of the explants. Results with BA (8.87 µM) and IAA at all three levels varied marginally. Inclusion of NAA in the medium not only induced lower response of multiple shoots but also resulted into heavy callusing at basal end of explants and shoots were hyperhydric.

On further subculture of these explants onto their respective media in test tubes did not improve multiple shoot number in majority of the media. It was observed that node region swelled and enlarged in size. When these explants were subcultured to glass bottles instead of test tubes, a dramatic increase in number of multiple shoots was observed at the end of 30 days. On an average 19.5 shoots per explant were recorded on MS basal medium supplemented with BA (8.87 µM) and IBA (1.48 µM) (Table 2). Also, a large number of shoot buds were observed in axils of multiple shoots (Fig. 1B, C and D). The poorest response was recorded on MS supplemented with BA (8.87 µM). MS alone without any growth regulator (control) did not show multiple shoot induction.

Due to its favorable response, BA has been the most commonly used cytokinin in grape tissue culture. BA in the range of 5-10 µM was found to be an effective growth regulator for induction of

shoots in grapevine cultures (Harris and Stevenson, 1982; Mhatre *et al.*, 2000). Lee and Wetzstein (1990) reported that higher BA levels (20, 30 and 40 µM) strongly inhibited shoot elongation, with few or no larger shoots in *Vitis vinifera* cv. Summit. They also reported that proportion of small (<1 cm) shoots increased with increased BA levels and cultures at higher BA levels had dense, unexpanded shoots with high mortality. Thomas (1997) obtained multiple shoots with poor elongation at BA (5 µM) in *Vinifera* cultivar 'Arka Neelamani'. He also observed that BA at higher level (10-20 µM) resulted into condensed shoots or undifferentiated growth. Chee and Pool (1985) reported that shoot proliferation of *Vitis* hybrid Remaily Seedless increased with increased concentration of BA (0-80 µM) reaching maximum at 5 µM. Hence optimization of BA concentration in the present study was considered a necessary step.

Table 2**.** Effect of growth regulators and subculture on induction and proliferation of multiple shoots in secondary nodal segments of Crimson Seedless

Medium composition (concentration, µM)	Explants showing multiple shoots (%)	Number of shoots per explant ± SD	
		(30 days)[1]	(90 days)[2]
MS+BA (8.87)	18.8	2.33±0.20	2.33±0.00
MS+BA (8.87)+IAA (0.57)	18.8	2.50±0.25	13.00±6.02
MS+BA (8.87)+IAA (1.14)	18.8	2.50±0.17	15.20±5.35
MS+BA (8.87)+IAA (1.71)	15.6	2.00±0.00	11.80±6.83
MS+BA (8.87)+IBA (0.49)	9.3	2.00±0.00	8.67±6.65
MS+BA (8.87)+IBA (0.98)	25.0	2.13±0.07	16.17±8.92
MS+BA (8.87)+IBA (1.48)	15.6	2.60±0.50	19.50±4.94
MS+BA (8.87)+NAA (0.54)	9.9	2.00±0.00	9.50±3.62
MS+BA (8.87)+NAA (1.07)	9.4	2.33±0.17	6.33±4.93
MS+BA (8.87)+NAA (1.61)	12.5	2.00±0.00	8.60±2.07
MS	0.0	0.00	0.00
LSD (*P*=0.05)	2.16	0.27	8.17
	*	*	**

* Significant at *P*=0.05; ** significant at *P*=0.01. [1] Explants - shoot clumps in test tubes; [2] Shoot clumps in bottles.

Table 3. Effect of BA and auxins on elongation of multiple shoots in Crimson Seedless

Medium composition (concentration, µM)	Number of shoots elongated per clump	Average height of elongated shoots (cm)
MS	3.60±2.73	1.70±1.85
MS+BA (2.22)	8.10±2.91	3.28±1.69
MS+BA (2.22) + IAA (0.57)	6.70±2.76	3.70±1.38
MS+BA (2.22) + IAA (1.14)	4.30±2.15	4.20±1.72
MS+BA (2.22) + IAA (1.71)	6.00±1.98	3.50±0.81
MS+BA (2.22) + IBA (0.49)	6.50±3.90	2.99±0.90
MS+BA (2.22) + IBA (0.98)	5.85±2.81	3.27±1.81
MS+BA (2.22) + IBA (1.48)	5.14±3.79	3.22±0.99
MS+BA (2.22) + NAA (0.54)	7.85±4.93	4.53±1.26
MS+BA (2.22) + NAA (1.07)	3.43±3.56	4.71±2.01
MS+BA (2.22) + NAA (1.61)	3.00±2.36	4.97±2.86
CD (P=0.05)	5.64	2.32
	**	**

** Significant at *P*=0.01. Culture vessels used: Glass bottles. Observations recorded after 30 days of inoculation.

Elongation of multiple shoots: Since multiple shoots induced were stunted in growth and were in form of clumps, it was necessary to define a medium for shoot elongation. MS basal medium supplemented with lower concentration of BA (2.22 µM) alone or in combination with IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM) was tested (Table 3). The least elongation was obtained on MS basal medium without any growth regulator (control). The maximum number of shoots (8.1 per clump) elongated (Fig. 1E) on MS with BA at 2.22 µM followed by 7.85 shoots per clump on MS with BA (2.22 µM) + NAA (1.61 µM). On these media, shoots grown could be separated easily from each other. Though comparatively higher elongation was achieved on media with NAA at all the three levels, however there was excessive callusing at base with adventitious roots, an effect undesirable for plant establishment. Similar to our findings, Mhatre *et al.* (2000) reported enhanced shoot elongation with addition of 1.14 µM IAA to the MS medium containing BA (2.22 µM) compared to the medium containing BA (2.22 µM) alone. From the published reports, it can be inferred that no fixed BA concentration was applicable for different stages of *in vitro* multiplication of different grapevine cultivars and concentrations need to be optimized for each cultivar.

***In vitro* rooting**: Since different auxins are documented to exhibit varying rooting response depending on cultivars, we investigated rooting response in shoots using MS half strength semi-solid medium gelled with agar (Fig. 1F) or gelrite (Fig. 1G) and also liquid medium with filter paper supports (Fig. 1H) with supplement of IAA (0.57-1.71 µM) or IBA (0.49-1.48 µM) or NAA (0.54-1.61 µM). Though rooting of *in vitro* shoots could be observed in varying percentages in agar or gelrite solidified or liquid media without auxins (control), however number of roots induced was fewer compared to media with auxins. It was also observed that with NAA, the number of roots was higher compared to media with IBA at same concentrations, which is in

Table 4. Effect of auxin pulse treatment on *ex vitro* rooting of shoots and plantlet establishment in Crimson Seedless

Treatment	Percentage of shoots rooted	Number of roots per shoot ± SD	Root length (cm) ± SD	Shoot length (cm) ± SD	Plants established (%)
Control	62.5	10.17±0.19	3.65±0.59	11.28±0.39	56.3
IAA 57.08 µM for 5 min	73.7	9.60±0.58	4.65±1.50	9.20±1.92	52.6
IAA 57.08 µM for 10 min	100.0	11.42±0.95	4.34±0.87	11.16±0.29	100.0
IBA 49.00 µM for 5 min	84.2	11.29±1.55	3.24±1.11	9.75±0.42	73.9
IBA 49.00 µM for 10 min	84.2	11.63±0.35	4.70±1.25	10.60±2.89	79.0
NAA 53.71 µM for 5 min	94.7	8.66±0.35	2.43±0.52	8.13±0.02	84.2
NAA 53.71 µM for 10 min	94.7	16.59±2.54	4.60±1.15	9.59±2.40	94.8
LSD (P=0.05)	17.8	1.86	1.70	2.60	12.4
	**	**	**	*	**

* Significant at *P*=0.05; ** significant at *P*=0.01. Observations recorded after 40 days of transfer to cups.

agreement with the findings of Helior *et al.* (1997). In the present study, the higher dose of auxin induced callusing and lead to poor establishment of the plants during hardening. Among the 3 auxins tested, NAA at 1.07 µM induced 100.0% rooting of shoots in all three conditions mentioned above and was found to be a better auxin for Crimson Seedless (data not shown). Survival of rooted shoots after hardening was 90.0%. Similar to our studies, Gray and Benton (1991) demonstrated that NAA at 1 µM incorporated in the media significantly increased the percentage of rooting, number of roots per shoot, and root length in three muscadine cultivars of grapevine. IBA is reported to be a better auxin for *in vitro* rooting of various clones of grapevine (Novak and Juvova, 1983) and in *vinifera* cv. Pinot noir (Helior *et al.*, 1997). Harris and Stevenson (1982) obtained better rooting with IAA (0.57 µM) in different clones of grapevines.

Though half strength MS medium devoid of growth regulators induced rooting in 84.1% shoots with an average of 5.2 roots per shoot, addition of auxin in the medium significantly improved the percent rooting, number of roots per shoot and survival percentage on potting.

***Ex vitro* rooting**: Experiments were carried out with *ex vitro* rooting to circumvent the *in vitro* stage, which takes about 3-4 weeks in culture. Elongated shoots were given pulse treatment of IAA (57.08 µM) or IBA (49.0 µM) or NAA (53.71 µM) for 5 or 10 min. Roots became visible through the transparent plastic cups (Fig. 1I) after 15 days of potting of shoots. Though rooting of shoots was observed in all the treatments, efficiency of response varied. The maximum response (100.0%) in terms of induction of roots and establishment of rooted shoots after potting was recorded with pulse treatment of IAA (57.08 µM) for 10 min (Table 4) (Fig. 1J). Gray and Benton (1991) in cultivars of muscadine grapes (*Vitis rotundifolia*) demonstrated that more shoots rooted *in vitro* than in *ex vitro* (77% vs. 46%), however, *ex vitro* technique was preferred, since acclimation of plants was achieved in lesser time and a major *in vitro* step was eliminated.

Hardening of plantlets: *In vitro* as well *ex vitro* rooted shoots and primary nodal segments with direct rooting could be hardened successfully by following Sachet technique. It was observed that covering of plantlets with polythene sachets for minimum of 4 weeks was very essential. Though top corners of bags could be cut after 2 weeks, however, complete removal of bags before 4 weeks caused scorching and drying of *in vitro* leaves.

From the present studies, it can be demonstrated that Crimson Seedless can successfully be propagated *in vitro* culturing single node segments on WPM without growth regulators where explants induced direct rooting at the base, initiated shoots and showed survival on potting. By this technique, hardened plantlets could be produced in 2 months. Another method is to induce multiple shoots in primary or secondary nodal segments and its proliferation on transfer to glass bottles after 60 days of culture in test tubes. By second route, plant production could be increased by many fold. Shoots could be rooted *ex vitro* by pulse treatment of auxins. This bypass one major *in vitro* stage and cuts down the cost and time of production. Hardening of plantlets could be achieved in a simple set up by Sachet technique. Thus, present procedure would be of immense help in commercial production of planting stock of this exotic variety of grapevine.

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