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A Critical Study in Vitro Plant Regeneration and Genetic Transformation Studies in Grapevine: Crimson Seedless

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Grape is the second most widely grown fruit crops of the world. It is grown under varied climatic conditions ranging from temperate to semi tropic and tropics. It is a woody perennial, cultivated in 90 countries, covering an area of about 19 million acres with Europe having the largest share (60%). Taxonomically, grapes are divided into two subgenera, Euvitis Planch. (2n=38) and Muscadinia Planch. (2n=40). Vitis vinifera belongs to the sub-genera Euvitis. The genus Vitis is broadly distributed between 25° and 50° N latitude in eastern Asia, Europe, the Middle East and North America. According to an estimate, 65.5 million tons of grapes were produced world over with a value of 144 billion pounds. In India, grape is grown on an area of 60,000 ha with a production of about 1.6 million tonnes, which comprises mainly of table. The paper will describe the results on plant regeneration system in Crimson Seedless by shoot organogenesis in in vitro leaves of the cultivar. Results on the influence of basal medium, explant type and various auxins and cytokinins on direct shoot organogenesis will be presented.

Key words : *Plant regeneration system, Crimson Seedless*

Introduction

Use of genetic engineering for crop improvement allows introgression of usefulagronomic traits without altering the other desirable features of a promising variety. This necessitates the availability of a rapid and efficient *in vitro* plant regeneration system. To date, regeneration of grape plants has been obtained by both shoot organogenesis (Martinelli *et al.,* 1996) and somatic embryogenesis (Torregrosa and Bouquet, 1996) from *in vitro* leaves. *De novo* shoot organogenesis via adventitious bud formation is preferred method of plant regeneration as the possibility of occurrence of somaclonal variations is minimum and the plantlets obtained from organogenesis are more uniform (Misra and Datta, 2001; Mujib, 2005). Moreover, plants raised through organogenesis were found to be better with regard to all economically important parameters compared to seed derived plants of white marigold (Misra and Datta, 2001).

Since the first report of adventitious bud formation *in vitro* in grapevine by Favre (1977), shoot organogenesis in varying frequencies from various plant parts has been documented (Reisch *et al.,* 1989; Mezzetti *et al.,* 2002). Rajasekaran and Mullins (1981) observed bud formation in the callus from internode segments of *Muscadinia rotundifolia*and hybrid derivatives of *vinifera* and *rupestris*. Use of leaf material, lamina or petioles as initial explants for regeneration of grapevines by the route of adventitious buds was earlier described by Reisch *et al*. (1989). Vilaplana and Mullins (1989) reported adventitious bud formation from hypocotyls and cotyledons of somatic embryos of Thompson Seedless, Grenache and *vinifera x rupestris* cv. Gloryvine and they reported that adventitious bud formation was influenced by genotype, tissue origin, stages of somatic embryos and level of embryo dormancy.

Explant source and BA concentration have been found to influence the adventitious shoot regeneration in mungbean (Mahalakshmi *et al.,* 2006) and *Prunus* (Matt and Jehle, 2005). Differential response of the *in vitro* leaves of different maturity levels, variablefrequency of shoot organogenesis between distal and proximal end of the leaves and among the leaves of different phyllotactic position have earlier been reported (Stamp *et al.,*1990).

Perez-Tornero *et al*. (2000) observed increase in percentages of shoot regeneration by two- fold in young expanding apricot leaves compared to the older ones. The differential response of two surfaces of *in vitro* leaves of sandalwood was observed by Mujib (2005). The influence of genotype on *in vitro* shoot organogenesis among different grapevine cultivars has been recorded in several studies (Favre, 1977; Vilaplana and Mullins, 1989; Stamp *et al.,* 1990; Martinelli *et al.,* 1996). Thus it is imperative to optimize conditions for regeneration system for each cultivar, clone or newly introduced variety. For adventitious shoot regeneration in many woody plants, urea based cytokinines like TDZ and CPPU were found to be more efficient than the adenine based cytokinines like BA, adenine sulfate, 2ip etc. (Leblay *et al.,*1990; Escalettes and Dosba, 1993; Sarwar and Sirvin, 1997; Hammatt and Grant, 1998). Induction of shoot regeneration on incorporation of auxins (IAA, IBA and NAA) in the medium has been reported (Yancheva *et al.,* 2003). Effectiveness of liquid culture to semi-solid culture for *in vitro* induction of adventitious budsin leaves of sandalwood was reported recently (Mujib, 2005). Colby *et al.* (1991) described histological findings of direct shoot organogenesis from *in vitro* leaves of grapevine hybrid French Colombard. Development of an efficient method of plant regeneration via organogenesis is a prerequisite for application of tissue culture to grapevine improvement through *Agrobacterium*-mediated gene transfer method. Hence, the present study was carried out to investigate the influence of different factors effecting direct shoot organogenesis including explant type, basal medium, different plant growth regulators, and liquid pulse treatment from *in vitro* leaves of grapevine cv. Crimson Seedless.

Materials and Methods

Plant material

In vitro leaves for the present study were collected from multiple shoot cultures of Crimson Seedless established from single node stem segments as mentioned in this paper. For induction of multiple shoots, secondary nodal segments were cultured on MS medium supplemented with BA (8.89 µM). Secondary nodal segments were obtained from shoots grown from primary nodal segments cultured on MS basal medium. After one month of culture in culture tubes, secondary nodal segments with induced multiple shoots were transferred to culture bottles containing the same medium composition for the further shoot proliferation. After first sub culture in the bottles, *in vitro* leaves were collected from the multiple shoot cultures by giving a cut at the mid of the petiole following the method described by Martinelli *et al*. (1996). The leaves were then injured at several places by surgical blade on lamina and petiole before inoculation. The leaf explants were inoculated in the petridishes with their abaxial (dorsal) surface in contact with the medium.

Influence of young and mature leaves

To optimize the type of explant effecting maximum organogenic response, young leaves measuring approximately 1.0 sq. cm and mature leaves cut into 1 sq. cm pieces were used for the present study. Both type of leaves were inoculated on half strength MS basal medium supplemented with BA (4.44 – 17.76 µM)

Influence of nutrient media

To evaluate the influence of basal medium on direct shoot organogenesis, *in vitro* leaves were inoculated on either ½MS or NN media supplemented with BA (0.89 – 22.22 µM).

Influence of cytokinins

To evaluate the influence of different cytokinins on shoot organogenesis, half strength MS basal medium supplemented with four cyokinins in a range of concentrations i.e.BA (2.22-17.76 µM) or TDZ (0.23-4.54 µM) or KIN (2.7-21.6 µM) or zeatin (0.23-4.56 µM) was used.

Influence of auxins

To test the influence of auxins on shoot organogenesis, four auxins namely IAA(0.09-0.26 µM), IBA (0.07-0.2 µM), NAA (0.08-0.24 µM) and NOA (0.07-0.22 µM) were supplemented to the media containing BA (4.44 µM).

Influence of liquid pulse treatment with growth regulators

In vitro leaves obtained from multiple shoot cultures were injured at several places onlamina and petiole by

surgical blade and given a liquid pulse treatment with a combination ofcytokinins and NAA (Table-5) for a period of 30 min. Leaves were then blotted gently on sterile filter paper and inoculated on half strength MS medium supplemented with BA (4.44
μM). The abaxial side of the leaves remained in contact with the medium. Untreated leaves were also transferred to the induction medium to serve as controls.

Each treatment in all the experiments had a minimum of ten replicates and the experiment was repeated at least three times. All the cultures were incubated initially in complete dark for 10 d and then shifted to 16 h photoperiod with a light intensity of 12.2 μmho cm^{-2} s^{-1} at 25±2°C. Leaves were shifted to fresh medium after 30 d of inoculation.Observations were recorded at regular intervals.

Shoot multiplication and rooting

Regenerated shoots or shoot clumps along with a part of mother explant (leaf / petioles) were transferred to MS medium supplemented with BA (8.89 μM) for multiplication and further shoot proliferation. Initially shoots were maintained in culture tubes for one month and later shifted to culture bottles containing the same medium composition. After another month on proliferation medium, multiple shoot clumps were transferred to MS medium supplemented with reduced levels of BA (2.22 μM) for elongation. Elongated shoots were rooted *in vitro* on half strength MS basal medium supplemented with NAA (1.07 μM).

Sucrose (3%) was added to all media gelled with 0.65% agar. All the growth regulators were added before autoclaving except zeatin, which was filter sterilized and added to the autoclaved media. Media after adjusting the pH to 5.8 were autoclaved at 121°C and 105 KPa pressure for 20 min.

Hardening of plantlets

Rooted shoots were transferred to plastic cups containing soil and sand (1:1) mixture and hardened as mentioned in the previous paper 3.1.2.7.

Results and Discussion

Adventitious shoots induced as early as 15 d after inoculation and continued until final observation recorded at 60 d. Shoots developed directly at cut ends of the petiole mostly(Fig.-1), and less frequently from ventral surfaces of the petiole (Fig.-1C) or cut surfaces, injured portions of leaf and midrib. In leaf explants, shoot induced mostly onventral side of the explant and towards the proximal end of the leaf as reported earlier by Stamp *et al.* (1990). Frequency of response varied depending on the type of explant, basal medium and growth regulators tested. Shoots induction occurred both, directly and viaintervening callus. Explants cultured in the media swelled before formation of adventitious shoots, an observation recorded earlier by Clog *et al*. (1990). In some treatments, after first subculture, leaves turned brown and became necrotic.

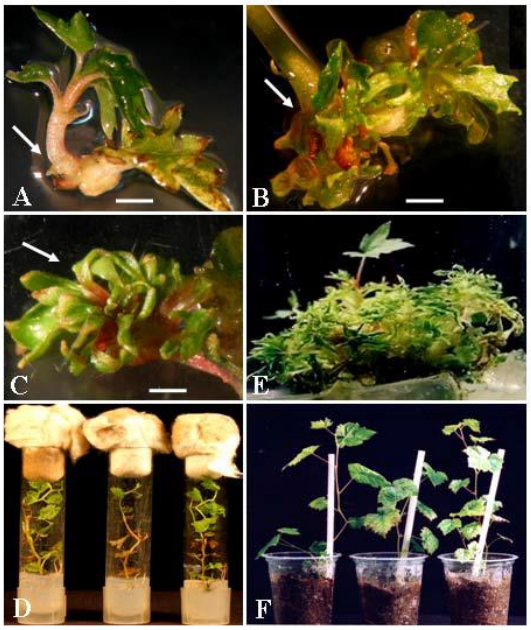

Fig.-1 : *De novo* shoot regeneration from *in vitro* leaves of CrimsonSeedless. A: Single shoot coming from cut end of the petiole (bar corresponds to 3 mm), B: Bunch of shoots from cut end of petiole (bar corresponds to 1.3 mm), C: Shoots from ventral side of petiole(bar corresponds to 650 µm), D: Shoots multiplied on MS+BA (8.89µM), E: Rooted shoots on ½MS + NAA (1.09 µM) and F: Hardenedplantlets.

Influence of leaf maturity on shoot organogenesis

Over all organogenic response was better in young whole leaves compared to mature cut leaves. The maximum response (16.67%) was observed in whole leaves cultured in half strength MS basal medium supplemented with BA (4.44 µM) (Table-1).

Table-1

Influence of leaf maturity on shoot organogenesis in Crimson Seedless

Explant	BA Conc. (µM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explants	No. of shoots per responded explants
			15 d	30 d	60 d			
Whole youngleaf	0	28	0	0	0	0.0	0	0
	4.44	30	0	5	5	16.67	1.2	2.2
	8.89	33	2	4	5	15.15	1.6	3.6
	13.32	35	3	5	5	14.29	1.2	3.6
	17.76	36	4	4	4	11.11	1.25	2.25
Mature leaf piece	0	24	0	0	0	0.0	0	0
	4.44	36	1	1	1	2.78	1.0	1.0
	8.89	35	1	3	3	8.57	1.67	4.0
	13.32	34	3	3	3	8.82	1.33	5.0
	17.76	36	4	4	4	11.11	1.25	2.75
SEM±						2.73	0.11	0.16
CD (p=0.01)						10.97	0.46	0.63
						**	**	**

** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference

Among the different BA concentrations tested, BA at 8.89 µM induced the maximum number of organogenic events per responded explant in both young whole leaf and mature leaf pieces. In case of mature leaf pieces, though the percent response was lower, however, number of shoots induced was higher (5 shoots/explant) compared to young whole leaves (3.6 shoots/explant) at BA 13.32 µM in the medium. Tissue maturity has been reported to influence the morphogenic competence and differentiation in crop plants (Sinnott, 1960) and the response may vary in different plant species. The phenomenon seems to be controlled by several intrinsic and external factors including physiological condition of the explants. In contrast to our results, Stamp *et al.* (1990) observed higher frequency of shoot organogenesis in bisected leaves compared to intact unwounded leaves.

Influence of two basal media and BA concentrations on shoot organogenesis

Between the two different basal media tested, half strength MS basal medium induced higher shoot organogenesis response compared to NN at similar concentrations of BA,. The maximum response (16.13 %) was recorded in ½MS basal medium supplemented with BA (4.44 µM) (Table-2). Different BA concentrations resulted in varying percentage of explants responding, however, number of organogenesis events per explant varied marginally. The maximum number of shoots (3.4) per explant was observed in ½MS withBA (8.89 µM).

Table-2

Influence of two basal media and BA concentrations on shootorganogenesis in Crimson Seedless

Basal medium	BA Conc. (µM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
½MS	0	28	0	0	0	0.0	0	0
	0.89	22	0	3	3	13.64	1.0	1.0
	2.22	33	0	2	3	9.09	1.0	1.33
	4.44	31	0	5	5	16.13	1.2	2.0
	8.89	33	2	4	5	15.15	1.5	3.4
	22.22	36	1	4	4	11.11	1.25	2.0
NN	0	36	0	0	0	0.0	0	0
	0.89	33	0	0	1	3.03	1	1.0
	2.22	35	1	2	2	5.71	1.5	1.5
	4.44	34	0	2	3	8.82	1.0	1.67
	8.89	35	1	2	4	11.43	1.25	3.0
	22.22	34	0	2	3	8.82	1.5	3.33
SEM±						1.11	0.13	0.15
CD (p=0.01)						2.35	0.53	0.61
						**	**	**

** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference.

Influence of cytokinins on shoot organogenesis

Among the three cytokinins (BA, TDZ and Zeatin) tested at different concentrations, half strength MS basal medium supplemented with BA (4.44 µM) induced shoot organogenesis in the maximum explants (16.67 %) with an average of 1.3 organogenic eventsand 1.6 shoots per explant (Table-3). Medium with BA at 8.89 µM resulted in 14.71 % response with an average of 1.6 organogenic events and 2.8 shoots /explant. Compared to BA, TDZ and Zeatin induced shoot organogenesis in lesser number of explants, however, average numbers of organogenic events and shoots per explants were higher. The maximum number of organogenic events (2.33) and number of shoots (3.67) per explant was observedin medium with TDZ 2.27 µM. Zeatin at 2.28 µM affected

the highest number of organogenic events (1.5) and number of shoots (4.25) per explant at 60d of observation (Table-3). Control explants did not show any organogenesis. In conformity with our findings, effectiveness of BA for induction of shoot organogenesis in leaves of different grapevine rootstocks was demonstrated by Clog *et al*. (1990).

Table-3
Influence of cytokinins on shoot organogenesis in Crimson Seedless

Cytokinin	Conc.(μM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
BA	2.22	32	0	3	3	9.38	1.33	1.33
	4.44	36	0	5	6	16.67	1.3	1.60
	8.89	34	2	4	5	14.71	1.6	2.8
	13.32	35	3	5	5	14.29	1.2	3.0
	17.76	36	4	4	4	11.11	1.25	1.5
TDZ	0.23	26	0	0	1	3.85	1.0	1.0
	0.45	26	1	1	1	3.85	2.0	2.0
	0.91	24	1	2	2	8.33	1.5	2.5
	2.27	25	2	3	3	12.00	2.33	3.67
	4.54	26	2	2	3	11.54	1.33	1.67
Zeatin	0.23	30	0	0	0	0.0	0	0
	0.46	32	1	2	2	6.25	1.5	2.5
	0.91	29	1	3	3	10.34	1.33	2.33
	2.28	34	2	2	4	11.76	1.5	4.25
	4.56	29	1	2	3	10.34	1.33	3.33
Control	--	28	0	0	0	0	0	0
SEM±						3.15	0.23	0.29
CD (p=0.01)						12.03	0.87	1.12
						**	**	**

* Basal medium – half strength MS
** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference.

Influence of auxins on shoot organogenesis

Four auxins (IAA, IBA, NAA and NOA) individually at different concentrationswere supplemented in the medium along with fixed BA (8.89 μM) with an aim if these can improve the rates of shoot organogenesis in *in vitro* leaves of Crimson Seedless. IAA (0.17 μM), IBA (0.15 μM), NAA (0.16 and 0.22 μM) and NOA (0.07 μM) induced higher responses compared to other concentrations of these auxins (Table-4). The maximum numbers of organogenic events (4.0) and shoots (2.67) per explant were recorded in the medium supplemented with IAA (0.26 μM) and NOA (0.22 μM), respectively (Table-4). The highest response (23.91 %) was observed in the medium supplemented with BA (8.89 μM) and NAA (0.24 μM) (Table-4).

Table-4
Influence of auxins on shoot organogenesis in Crimson Seedless

BA Conc. (µM)	AuxinConc. (µM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
4.44	--	33	2	4	5	15.15	1.6	2.4
	IAA 0.09	36	2	2	4	11.11	1.25	2.0
	0.17	36	3	4	7	19.44	1.14	1.57
	0.26	36	3	3	3	8.33	4.0	2.33
	IBA 0.07	34	3	3	3	8.82	1.33	2.0
	0.15	34	3	4	5	14.71	1.6	2.2
	0.22	34	3	3	3	8.82	1.0	1.33
	NAA 0.08	48	4	4	5	10.42	1.2	2.0
	0.16	66	8	10	14	21.21	1.43	2.29
	0.24	46	6	11	11	23.91	1.45	2.27
	NOA 0.07	24	3	5	5	20.83	1.2	1.8
	0.15	24	2	3	3	12.50	1.67	2.33
	0.22	24	2	3	3	12.50	1.67	2.67
SEM±						1.90	0.13	0.18
CD (p=0.01)						3.38	0.49	0.69
						**	**	**

** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference.

Cheng and Reisch (1989) reported the importance of auxin in the regeneration of *in vitro* leaf explants of grapevine cv. Catawba. They also demonstrated that altering BA levels in the propagation medium played a crucial role in regeneration of petiole explants and light had an inhibitory effect on *in vitro* regeneration process. Torregrosa and Bouquet (1996) found NAA to be more effective auxin compared to IBA for induction of shoot organogenesis in leaves of grapevine. NAA at lower concentrations along with BA induced higher responses. Also they observed that organogenesis response was better in *in vitro* leaves obtained from axillary shoot cultures grown in medium with BA compared to leaves collected from rooted plantlets growth without growth regulators.

Influence of plant growth regulator pulse treatment on shoot organogenesis

In another experiment with an aim to improve the response of shoot organogenesis, *invitro* leaves were given pulse treatment of growth regulator for 30 min and thereafter culturedin ½MS + BA (8.89 µM). Compared to continuous culture of explants on agar media, liquid pulse treated leaves showed higher response in terms of percentage of organogenesis and number of adventitious shoots induced per explant. Among the four cytokinins used, KIN and TDZ were comparatively more effective. Liquid pulse treatment of leaves with KIN (18.58 µM) + NAA (2.7 µM) induced direct shoot organogenesis in maximum number of leaves (70.83%), followed by 62.5% either in KIN (18.58 µM)+NAA (1.08 µM) or TDZ(4.54 µM)+NAA (2.70 µM) (Table-5). In case of media supplemented with TDZ or KIN, the organogenic response increased with the increase of NAA concentration from 0.27 to 2.7 µM. The results on number of organogenic events per responded leaf were found to be non-significant. Single organogenic event was seen per leaf in most of the treatments and with a maximum of five organogenic events were observed in a few explants. Whereas, average number of organogenic events per responded explant ranged from 1.0 to 2.0. Average number of shoots obtained from each leaf varied significantly among the treatments. Number of shoots per responded leaf was maximum (4.33) in case of leaves given pulse

of BA (17.75 µM) + NAA (0.54 µM) followed by 3.58 shoots /explant in case of BA (17.75 µM) + NAA (1.08 µM). In general, number of shoots per responded leaf was higher in BA treatments as compared to other cytokinins.

Table-5

Influence of plant growth regulator pulse treatment on shootorganogenesis in Crimson Seedless

Cytokinin Conc. (µM)	NAA Conc. (µM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing shoot organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
BA (17.76)	0.27	36	2	5	5	13.89	1.60	3.8
	0.54	36	3	5	6	16.67	2.00	4.33
	1.08	35	8	12	12	34.29	1.75	3.58
	2.70	36	5	6	8	22.22	1.00	2.25
	5.40	36	5	5	5	13.89	1.00	1.60
TDZ (4.54)	0.27	26	5	6	6	23.08	1.00	1.33
	0.54	24	9	13	13	54.17	1.38	3.00
	1.08	25	12	14	14	56.00	1.36	2.50
	2.70	24	15	15	15	62.50	1.40	2.47
	5.40	24	5	6	6	25.00	1.00	1.50
KIN (18.58)	0.27	25	5	8	8	32.00	1.25	2.13
	0.54	26	9	12	15	57.69	1.20	2.20
	1.08	24	12	15	15	62.50	1.40	2.67
	2.70	24	12	15	17	70.83	1.47	2.41
	5.40	24	6	7	7	29.17	1.00	1.43
Zeatin (4.56)	0.27	24	4	4	4	16.67	1.25	2.00
	0.54	24	5	6	6	25.00	1.00	1.67
	1.08	24	5	7	7	29.17	1.29	1.86
	2.70	24	5	5	5	20.83	1.00	1.80
	5.40	24	2	3	3	12.50	1.00	1.67
Control	-	36	2	3	5	13.89	1.20	1.80
SEM±						5.14	0.55	0.50
CD (p=0.01)						8.91	0.96	0.87
						**	NS	**

Leaves after pulse treatments were cultured on ½MS + BA (4.44 µM)

** Significant at 1% level, NS–Non-significant, SEM – Standard error of mean, CD –Critical difference.

Chen *et al*. (2001) reported maximum adventitious shoot formation from internode explants of *Adenophora triphylla* in the medium supplemented with BA and NAA. Contrary to these results, Zeatin was found to be more effective growth regulator compared to BA in tubers of two wild Potato cultivars (Kikuta and Okazava, 1982; Anjum and Ali, 2004). Cytokinins are known to induce axillary and adventitious shoot formation (Madhulatha *et al.*,2004). Leaves without any pulse treatment showed shoot organogenesis in only 13.89% explants with an average 1.8 shoots per responded explant. Higher levels of NAA induced callusing and organogenesis occurred via callus. Shoots regenerated via callus appeared to behyperhydric and showed poor growth on sub culture. Such shoots were short and did not multiply. In conformity with our results, lower frequency of shoot regeneration and

lower plant survival rate was reported in shoots developed via callus in mungbean (Gulati and Jaiwal, 1990). However, Martinelli *et al.* (1996) had no problems in obtainment of multiple shoots, even if organogenesis was affected via callus phase.

In the present study, pulse treatment of *in vitro* leaves with plant growth regulators significantly improved the percentage of leaves showing direct shoot organogenesis and number of shoots induced per explant. Higher regeneration response with liquid pulse treatment has earlier been reported for *in vitro* propagation of banana cv. Nendran (Madhulatha *et al.*, 2004) and adventitious shoot induction in epicotyl explants of Azuki bean(Mohamed *et al.*, 2006). Drake *et al.* (1997) reported shoot regeneration in 62.5% of cotyledonary explants of Sitka Spruce given liquid pulse treatment of BA (400 µM) for 2 h. Changes in physiological state of the explants in liquid culture due to their complete immersion in the media with increased absorption of growth regulators has been reported to be a reason for improved organogenesis response (Mujib, 2005; Mohamed *et al.*, 2006).

Adventitious shoots developed mostly from injured regions of laminae and petiole and largely on ventral (adaxial) surface. Number of adventitious buds formed on petiole varied from 1-6 via callus at cut ends. Bud formation was more prolific in lamina and was associated with callus at cut ends of the vein and midribs. This differential morphogenetic response could be due to variation in physiological status of different parts of a leaf (Mujib *etal.*, 1996). Takeuchi *et al.* (1985) reported that wounding of stem segments of *Torenia* significantly improved the formation of adventitious buds. Mujib (2005) reported that frequency of shoot organogenesis and adventitious bud formation was higher on ventral surface of the leaves and liquid medium was more effective compared to semi-solid. In another study, pulse treatment of *in vitro* leaves with TDZ at 100 µM for just 30 s induced shoot proliferation in Petunia explants (Fellman *et al.*, 1987).

Regenerated shoots and shoot clumps could be proliferated on MS basal medium supplemented with BA (8.89 µM) (Fig.-1D). After 60 d of culture, the shoot clumps were transferred to MS + BA (2.22 µM) for shoot elongation. Elongated shoots when transferred to ½MS + NAA (1.09 µM), showed 100% rooting *in vitro* (Fig.-1E) and plantlets showed 90% survival after hardening (Fig.-1F).

Histological studies revealed the development of meristematic regions in the petiole and leaf surfaces, which later developed into shoot meristems. Shoots appeared to have developed from sub-epidermal parenchyma cells (Fig.-2).

Martinelli *et al*. (1996) reported shoot regeneration via direct organogenesis without callus from the cut surfaces of the petiole in several grapevine cultivars and rootstocks. However, in *Vitis armata*, V. *simpsonii* and sultana moscato regeneration occurred via callus phase. In few cases direct regeneration occurred from midrib of the leaf. Tang and Mullins (1990) observed adventitious bud formation from both lamina and petiole. Among the genotypes they tested, frequency of response was higher in St. George, Thompson Seedless and Niagara. Also, they found that NN media containing BA (10 µM) and NAA (0.05 – 0.10 µM) was the most effective in inducing adventitious buds in all the genotypes where as 2,4-Dinduced only callusing.

Stamp *et al*. (1990) observed that the frequency of shoot organogenesis depended on the age of leaves. Younger leaves collected from *in vitro* shoots resulted in higher percentage of responses, which decreased with increasing leaf maturity. Whole leaves along with petiolar stubs have earlier been used for adventitious bud induction and shoot regeneration byTorregrosa and Bouquet (1996). They observed that for adventitious bud formation, it was essential to include BA in the medium and a supplement of NAA (0.01 µM) further enhancedthe response. Age of leaves had influence on regeneration capability. Leaves collected from shoot cultures maintained in the medium with BA (4.44 µM) exhibited maximum efficiency of shoot organogenesis (Torregrosa and Bouquet, 1996).

Proliferation of adventitious shoots formed from internode explants of *Adenophora triphylla* was better in liquid MS basal medium supplemented with BA (17.78 µM) than inthe semi-solid medium (Chen *et al.*, 2001). Earlier studies on regeneration of woody plants from leaves underlined the crucial role of growth regulators. In many studies TDZ was found to be more responsive in regeneration of adventitious shoots compared to BAP (Hammatt andGrant 1998; Matt and Jehle, 2005). However, in another study, BA was found to be more effective than TDZ

for induction of adventitious shoots from sweet and sour cherry leaves (Tang *et al.*, 2002). In contrast to these reports, Yang and Schmidt (1992) did not observe anydifference between these two cytokinins. Earlier TDZ in combination with NAA induced higher organogenesis response in *in vitro* leaves of sweet cherry cultivars (Bhagwat and Lane2004; Matt and Jehle, 2005) and increasing NAA concentrations adversely effected the regeneration frequency which corroborates our findings. Sul and Korban (2004) did not find any complementary effect of NAA added to the media containing either BA or TDZ on directshoot organogenesis in cotyledons of *Pinus* species.

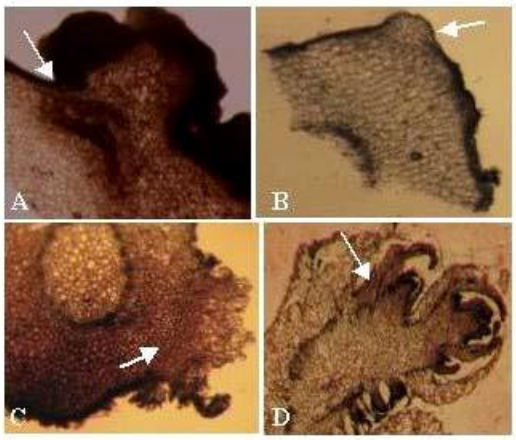

Fig.-2. Leaf (A) and petiole (B-D) showing *de novo* shoot formation.SM: shoot meristem; SP: shoot primordium. Bar = 2 mm

Diffusible growth factor present in the leaves and other explants responsible for organogenesis has been assumed to be an auxin like substance that, in the presence of cytokinin, activates the totipotent cells for bud formation as reported in soybean (Cheng *et al.*, 1980) and *Brassica juncea* (Shrama *et al.*,1991). Wounding of tissue was found to enhance the organogenesis response as manifested in form of number of shoots from the cut surfaces in the present study. The possible reason could be the release of endogenous growth regulators affected by wounding (Smith and Krikorian, 1990). In another study, wounding disturbed the ability of tissues to regulate K^+ ion exchange leading to increased osmotic potential of cells and the generation of an electrical field across the explant which, in turn controls the organogenesis (George and Sherrington, 1984).

Conclusion

From the present study, it was found that cytokinins played an important role in induction of shoot organogenesis in *in vitro* leaves of Crimson Seedless and the response could be further improved by supplementing auxins in the regeneration medium. Further, efficiency of direct shoot organogenesis could be substantially improved by pulse treatment of explants with growth regulators before culturing them on semi-solid medium. In the present study use of leaves obtained from *in vitro* shoots were preferred as explants for shoot organogenesis instead of leaves from field grown vines, since young and contamination free material, independent of seasonal variations was available round the year.

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