Research Paper

In Vitro Regeneration in *Celastrus paniculatus* Willd: Influence of Explant, Gelling Agent and Carbon Source

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Adventitious shoot regeneration from internodal explants harvested aseptically from mature node derived *in vitro* multiplying shoot cultures of *Celastrus paniculatus* was influenced by internode length, its position at source shoot, gelling agent and carbon source. Regeneration capacity (number of shoots per explant) of internode segments was increased with increasing internode length up to 1.5 cm. However, internode segments of 1.0 cm length produced 14.67 shoots per explant measuring average height of 1.89 cm on standard regeneration medium containing 4.44 µM 6-benzyl aminopurine where unwanted callus formation was not observed. Among different positions of internodes, 8th (basal) internode segment proved to be the best explant where maximum number (18.67) of shoots were produced per explant. Crude agar (General Purpose agar; BDH) proved to be the most effective gelling agent in differentiation of maximum number of adventitious shoots where the shoots attained an average height of 3.75 cm. Incorporation of different carbon sources in the range of 1-5 % in the regeneration medium evoked varied responses in terms of differentiation and elongation of adventitious shoots. However, glucose at its 4.0% concentrations was most effective carbon source where maximum number of elongated shoots ($\geq$ 1.0 cm in height) were produced. On this concentration, an average of 10 elongated shoots were produced per explant where all the cultured internode explants responded favourably in terms of shoot regeneration and their elongation.

Key Words: Medicinal plant; Internode; Shoot bud Differentiation; Shoot bud elongation

Introduction

Celastrus paniculatus Willd. (family: Celastraceae) commonly known as 'Malkangni' or 'Jyotishmati' is a large woody climbing shrub which occurs in the hilly parts of India upto an altitude of 1200 m. The plant is valued for its immense medicinal properties. Oil obtained from the seeds is used for rheumatism, gout and beriberi [1]. The oil is also used to promote intelligence and to sharpen memory [2]. The plant is known to contain a number of useful compounds. These include malkangunin (sesquiterpene polyesters), celapanin, celapanigin, celapagin (sesquiterpene alkaloids) and celastrol, pristimerin, zeylasterone (quinine-methide and phenolic triterpenoids) [3]. Since the plant has been listed as a threatened species [4], *ex situ* conservation efforts have been devoted through micropropagation using different pathways [5-9]. These reports deal mainly with the effect of plant growth regulators during *in vitro* growth and development. The present investigation was undertaken to examine the effect of explant length and its position at source shoot (positional effect), gelling agents, and type and concentration of carbon source (sugars) on *in vitro* shoot regeneration from internode explants in *Celastrus paniculatus* in order to develop a highly reproducible and improved regeneration system which would be useful in both large scale multiplication and genetic transformation studies in this plant species.

Materials and Methods

In vitro shoot cultures were established using mature nodes obtained from field grown plant of *Celastrus paniculatus* on Murashige and Skoog (MS) [10] medium containing 3.0% sucrose, 0.8% agar and supplemented with 2.22 µM BAP as per the method described by Bilochi [5]. These *in vitro* multiplying shoot cultures were used as the source of internode explants for the present studies. Four independent experiments (Experiment 1, 2, 3 and 4) were conducted to examine the influence of various factors (single factor was studied per experiment) on regeneration potential of internode explants obtained from *in vitro* multiplying shoot cultures of *C. paniculatus.* In Experiment 1, internode segments of different length (i.e. 0.5, 1.0, 1.5 cm), randomly selected from 3rd to 4th position from the source shoot were used as explants. For the study of positional effect of internodes (Experiment 2) on regeneration potential, internodes measuring 1.0 cm in length were harvested aseptically on Laminar Flow Clean Air Bench from 3rd to 8th positions (i.e. from shoot tip towards its base) of source shoot. The internode explants were cultured on standard regeneration (SR) medium consisting of MS salts, 0.8% agar (Hi-media), 3.0% sucrose (Hi-media) and supplemented with 4.44 µM BAP. In case of experiment 3 and 4, internode segments measuring 1.0 cm in length were randomly collected from 3rd to

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4th positions of the source shoot and used as the explants. In Experiment 3, the explants were cultured on SR medium solidified with different gelling agents *viz;* 0.8% Agar (Bacteriological grade, Hi-media; control), 0.8% Crude agar (General Purpose agar, BDH), 0.2% Phytagel™ (Sigma), 3.0% Guar-gum (Satyam Guar-gum Industries, Jodhpur, India), 3.0% Isubgoal (Gujarat Sat-Isubgoal Factory, Gujarat, India) and 13.0% Sago. While in case of Experiment 4, the internode explants were cultured on the SR media gelled with 0.8% agar and supplemented with different concentrations (1-5%) of various sugars viz; Glucose (BDH), Fructose (Hi-media), Sucrose (Hi-media), Maltose (DIFCO), and Lactose (BDH). In all experiments, the explants were inoculated horizontally on the slanted medium in culture tubes (25 x 150 mm, Borosil, India) and plugged with non-absorbent cotton. The pH of the media was adjusted to 5.8 prior to autoclaving at 1.06 kg cm^{-2} for 15 min. All the cultures were kept under controlled conditions of temperature (28 ± 2°C), light (45 µmol m^{-2} s^{-1} for 16 h per day provided by fluorescent cool white tubes) and 50-60% relative humidity. The explants were regularly subcultured on to the same fresh medium after every 21 days. The response has been expressed in terms of average number of shoots per explant and average shoot height in Experiment 1, 2 and 3. For Experiment 4, average number of shoots (morphologically differentiated shoot buds ≥ 0.5 cm in height) and elongated shoots (morphologically differentiated shoot buds ≥ 1.0 cm in height) regenerated per explant, average height of elongated shoots and per cent explants with elongated shoots were recorded after 63 days of cultures.

Each treatment consisted of six replicates with one explant per culture tube and each experiment was repeated twice. The data being similar only one representative experiment was used for statistical analyses.

Statistical Analysis: The experiments were conducted in completely randomized design (CRD). The data were analyzed by CRD to identify best treatment combination.

Results

The internode explants cultured on SR medium showed adventitious shoot differentiation only from their surfaces. None of the shoot regeneration event was observed from the cut ends of cultured internode explants in the present investigation. The explant length and its position (internode explants obtained from different regions of source shoot) influenced adventitious shoot regeneration potential of internode explants in *C. paniculatus* cultured on SR medium. The number of adventitious shoots differentiated per explant increased with the increasing length of internode segments (Table 1). Although, the internodal segments of 1.5 cm in length produced maximum number of shoots (16.17) per explant but a small amount of unwanted callus was induced at both cut ends of horizontally placed internodes. In this respect, internode segments of 1.0 cm in length proved to be the best as they produced 14.67 shoots per explant measuring an average height of 1.89 cm without an intervening callus phase (Table 1). No significant differences were observed in the average shoot height when internode segments of different length were cultured on SR medium (Table 1). In the present investigation, differences in the regeneration potential were observed when the internodal explants collected from different regions of the source shoot were cultured on SR medium. The number of shoot buds regenerated per explant increased from 3rd to 8th internode segments (i.e. from shoot apex to its base) (Table 2). However, the 8th internode segment proved to be the best explant as it produced maximum number of shoots (18.67) per explant which measured 1.59 cm in height.

Differences in regeneration capacity (average number of shoots per explant) and shoot height were observed when the SR medium was solidified with a variety of gelling agents. Explants cultured on the SR medium solidified with 0.8% agar (control) produced an average of 14.67 shoots with an average height of 1.89 cm (Table 3). This response was improved by incorporation of either Phytagel™ or crude agar as

Table 1. Effect of explant length on *in vitro* shoot regeneration from internode segments in *Celastrus paniculatus*

Internode length (cm)	Average number of shoots* [explant^{-1}] ±SD	Average shoot height (cm)±SD
0.5	12.17^{c*1}±0.75	1.96±0.40
1.0	14.67^{b}±1.03	1.89±0.22
1.5	16.17^{a}±0.75	1.98±0.26
SEM	0.35	0.12
CD (5%)	1.05	NS
CV	5.97	15.74

* Shoot buds more than 0.5 cm in height were counted as shoots
*1 Means followed by different letters differ significantly at P≤ 0.05
SEM- standard error of means; CD- critical difference; CV- coefficient of variance; NS- Not significant

Table 2. Effect of explant position on *in vitro* shoot regeneration from internode segments in *Celastrus paniculatus*

Internode number (from shoot apex)	Average number of shoot* [explant^{-1}] ±SD	Average shoot height (cm)±SD
3rd	13.00^{c*1}±1.41	1.03^{b}±0.25
4th	16.17^{b}±1.17	1.26^{b}±0.22
5th	16.50^{b}±1.22	1.38^{ab}±0.28
6th	17.50^{ab}±1.38	0.95^{c}±0.15
7th	18.17^{a}±1.17	1.34^{ab}±0.12
8th	18.67^{a}±1.21	1.59^{a}±0.36
SEM	0.52	0.10
CD (5%)	1.49	0.29
CV	7.59	19.28

* Shoot buds more than 0.5 cm in height were counted as shoots
*1 Means followed by different letters differ significantly at P≤ 0.05
SEM- standard error of means; CD- critical difference; CV- coefficient of variance

gelling agent in the medium in place of agar. However, significant increase in number of shoots (17.0) per explant was obtained on the medium solidified with crude agar (Table 3). These shoots attained an average height of 3.75 cm. Reduction in regeneration capacity was recorded on the media gelled with either Sago, Guar-gum or Isubgoal. However, Guar-gum promoted shoot elongation as equally as observed on Phytagel^{TM} where shoots measured 4.0 cm in length (Table 3).

Type and quantity of carbon source influenced in vitro differentiation and elongation of adventitious shoots in *C. paniculatus.* Internodal explants cultured on SR medium containing 3.0% sucrose (control) produced 14.67 shoots per explant where only 6.0 elongated shoots measuring 2.90 cm in height were obtained after 63 days of culture (Table 4). This medium favoured both differentiation and elongation of adventitious shoots in all the explants cultured. Any increase or decrease in the sucrose concentration of 3.0% diminished this response. Addition of various carbon sources in place of sucrose in the medium evoked varied response. The regeneration response was increased when the medium was supplied with either maltose, glucose or fructose in place of sucrose as carbon source. However, glucose at its 4.0% concentration proved to be the best carbon source where maximum number (10.0) of elongated shoots were produced per explant (Table 4). This medium favoured the development of elongated shoots from all the cultured internodes. On this concentration of glucose (4.0%), an average of 21.0 shoots were produced per explant. Although, fructose at its 5.0% concentration induced maximum number (26.0) of adventitious shoots per explant but these shoots remained stunted in most of the cultured internode explants even after 63 days of culture. Also, a small amount of callus was observed at the explant surface on all the treatments (1-5%) of fructose tested. Incorporation of lactose in the medium adversely affected the regeneration response in internode explants.

Table 3. Effect of different gelling agents on *in vitro* shoot regeneration from internode explants in *C. paniculatus*

Gelling agent type	Average number of shoot* [explant^{-1}] ±SD	Average shoot height (cm)±SD
Agar	14.67^{b*1}±1.03	1.89^{b}±0.17
Crude agar	17.00^{a}±1.10	3.75^{a}±0.45
Phytagel^{TM}	15.00^{b}±1.26	4.00^{a}±0.88
Guar gum	10.67^{c}±0.82	4.00^{a}±0.90
Isubgol	11.17^{c}±1.72	2.00^{b}±0.37
Sago	8.17^{d}±1.17	1.44^{b}±0.35
SEM	0.50	0.24
CD (5%)	1.43	0.69
CV	9.51	20.65

* Shoot buds more than 0.5 cm in height were counted as shoots
*1 Means followed by different letters differ significantly at P≤ 0.05
SEM- standard error of means; CD- critical difference; CV- coefficient of variance

Table 4. Effect of carbon source on in vitro shoot regeneration from internode explants in Celastrus paniculatus

Carbon source	Concentration of carbon source (in per cent)	Number of shoot* [explant^{-1}] ±SD	Number of elongated shoots [explant^{-1}]** ±SD	Average height of elongated shoots (cm)** ±SD	Explants with elongated shoots (in per cent)
Sucrose	1.0	3.00^{l*1} ±0.89	NE	NE	NR
	2.0	9.17^{gh} ±2.14	1.33^{g*1} ±0.52	1.17^{e*1} ±0.24	100
	3.0	14.67^{ef}±1.03	5.83^{b} ±0.75	2.90^{a} ±0.43	100
	4.0	9.50^{g} ±1.76	4.60^{be}±0.89	2.90^{a} ±0.24	83.33
	5.0	6.67^{ij} ±1.21	2.50^{ceg}±0.71	1.50^{cde}±0.71	33.33
Maltose	1.0	4.00^{kl} ±1.26	NE	NE	NR
	2.0	8.67^{ghi}±1.63	NE	NE	NR
	3.0	17.33^{cd}±1.51	2.75^{fg}±0.96	1.25^{de}±0.24	66.67
	4.0	16.33^{de}±1.37	3.50^{edf}±1.05	1.83^{bc}±0.18	100
	5.0	15.00^{ef}±1.41	4.33^{cef}±1.03	1.63^{cd}±0.30	100
Glucose	1.0	2.50^{l} ±1.05	NE	NE	NR
	2.0	12.83^{f}±2.23	2.0^{dg}±0.00	2.08^{abc}±0.11	33.33
	3.0	19.50^{bc}±2.59	6.20^{b}±1.64	2.22^{b}±0.37	83.33
	4.0	21.00^{b}±2.37	10.17^{a}±1.47	1.92^{bc}±0.13	100
	5.0	16.83^{de}±1.72	9.67^{a}±1.21	2.17^{b}±0.43	100
Fructose	1.0	5.67^{jk}±1.03	NE	NE	NR
	2.0	16.17^{de}±2.40	6.0^{bcd}±1.41	3.25^{a}±0.35	33.33
	3.0	21.17^{b} ±2.79	4.0^{cedf}±1.41	2.07^{bc}±0.12	66.67
	4.0	25.67^{a} ±1.75	4.0^{cedf}±1.22	1.33^{de}±0.41	83.33
	5.0	26.50^{a} ±2.43	6.0^{bcd}±1.41	1.58^{bce}±0.59	33.33
Lactose	1.0	3.33^{l} ±0.82	NE	NE	NR
	2.0	3.33^{l} ±1.03	NE	NE	NR
	3.0	7.00^{hij}±1.41	2.0^{dg}±1.41	1.50^{ce}±0.14	33.33
	4.0	7.50^{ghij}±1.05	2.0^{dg}±0.00	1.0^{ce}±0.14	33.33
	5.0	6.00^{jk} ±1.26	NE	NE	NR
CV		14.25	22.77	16.60	

** Shoots measuring more than 1.0 cm in height were designated as elongated shoots and only the responsive explants were considered to obtain means of elongated shoots.

* Shoot buds more than 0.5 cm in height were counted as shoots; *1 Means followed by different letters differ significantly at P≤ 0.05; CV- coefficient of variance; NE- no elongation; NR- no response.

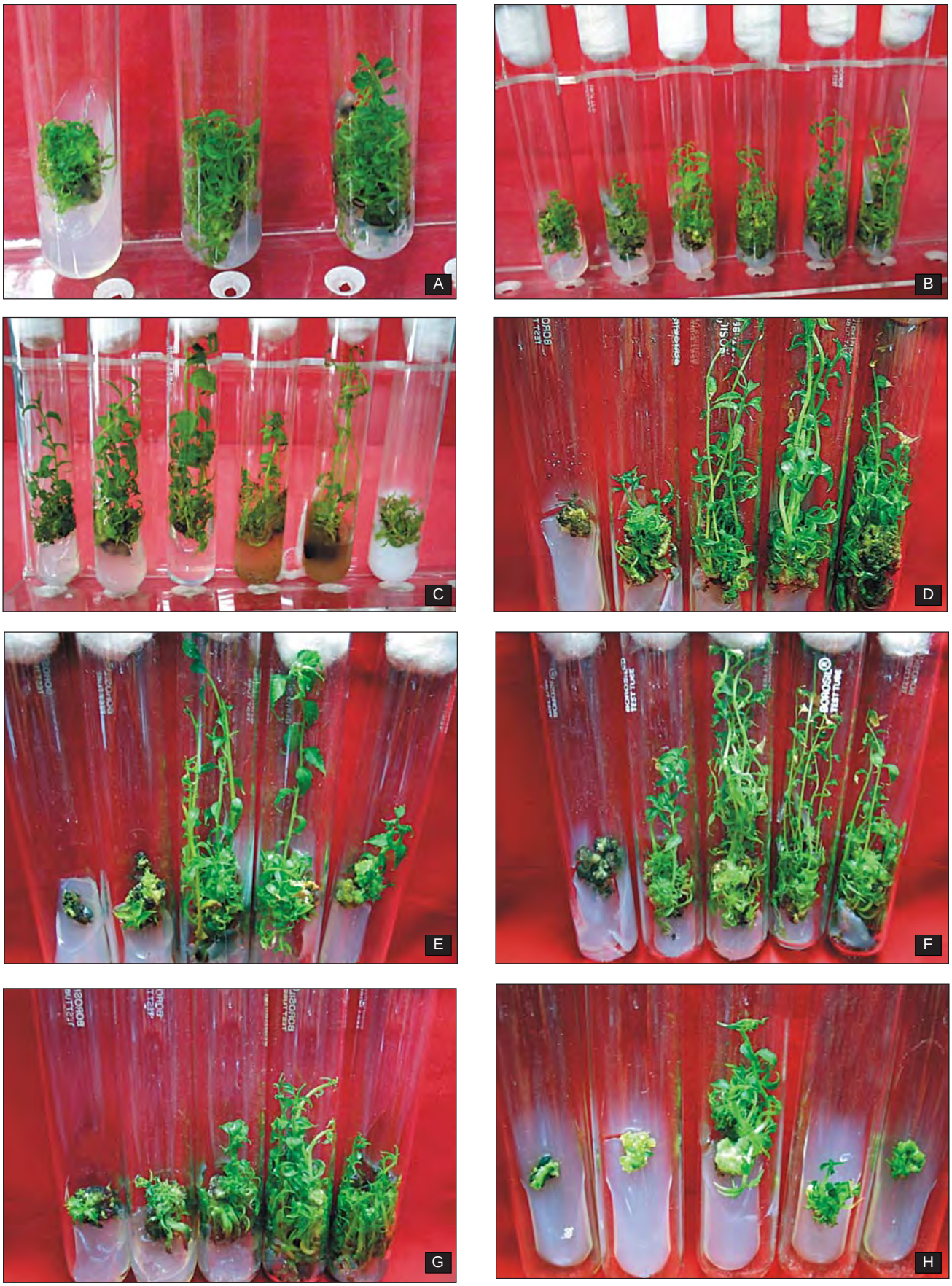

Fig. 1: *Effect of explant length* (A) *(a) 0.5, (b) 1.0, (c) 1.5 cm; Its position* (B) *(a) 3rd (b) 4th (c) 5th (d) 6th (c) 7th (f) 8th internode from shoot apex; gelling agents* (C) *(a) agar, (b) crude agar, (c) PhytagelTM, (d) isubgol, (c) guar-gum, (f) sago and carbon sources: sucrose* (D), *maltose* (E), *glucose* (F), *fructose (G), lactose* (H) *(a) 1, (b) 2, (c) 3, (d) 4, (c) 5% on adventitious shoot regeneration from internode explants of C. paniculatus*

Differentiated shoots remained stunted at 5.0% concentration of lactose, 2.0% concentration of maltose and lactose and 1.0% concentration of all the sugars tested even after 63 days of cultures.

Discussion

The data presented here showed that the regeneration response of internode explants in *C. paniculatus* was influenced by various factors studied. Increased regeneration capacity was noticed with increasing length of internode explants (Table 1). This might be explained as it provided maximum number of cells to induce meristematic activity under the direct influence of plant growth regulators present in the medium which in turn resulted in higher number of adventitious shoot buds development. However, a little amount of unwanted callus was also noticed in longest internode segments. Hence, in all the subsequent experiments internodes measuring 1.0 cm in height were used to avoid callus formation. The influence of explant position (positional effect) on morphogenesis has been studied in several cases [11, 12, 13]. In the present investigation, the regeneration capacity increased from 3rd (apical) to 8th (basal) internode segment (Table 2). These significant differences in regeneration potential may occur due to a gradient of phytohormone and/or polyamines existing within the same system [14, 15]. The basipetal increment of regeneration response has also been reported [16, 17].

Gelling agents used to solidify plant growth medium can contain considerable quantities of mineral nutrients that have been reported to affect plant growth. In the present case, 14.67 shoots measuring average height of 1.89 cm were produced from internode explants cultured on SR medium containing 0.8% agar and 3.0% sucrose (control) (Table 3). This response was improved when the medium was solidified with either Phytagel^{TM} or crude agar. However, crude agar produced maximum number (17.0) of shoots measuring an average height of 3.75 cm (Table 3). The beneficial role of Phytagel^{TM} in tissue culture has been reported in *Bacopa monnieri* [18]. Influence of agar type on shoot proliferation was investigated by Singha [19]. Agar quality has always been correlated with high gel strength [20]. Thus the higher shoot regeneration capacity and shoot length on crude agar gelled medium rather than agar (bacteriological grade) might be due to its higher nutritional value and low gel strength (due to low quality) which may result in increased water availability and diffusion of nutrients to the explants cultured. The use of Guar gum [21,22,23], Isubgol [24, 25] and Sago [5, 26] as an alternative gelling agent has been demonstrated in several reports. In the present investigation, addition of either Guar gum, Isubgol or Sago as a substitute of agar resulted in the reduced number of shoots per explant. However, Guar gum promoted elongation of regenerated shoots as equal as observed with Phytagel^{TM}. The promontory effect of Guar gum on *in vitro* shoot proliferation has been reported in *T. bellerica* [27], Strawberry [28] and tobacco [23].

The influence of type and quantity of carbon sources (sugars) on the morphogenesis has been reviewed extensively [29, 30, 31]. Internode explants cultured on SR medium containing 3.0% sucrose produced an average of 14.67 shoots along with 6.0 elongated shoots per explant (Table 4). Further increase in sucrose concentration resulted into poor shoot regeneration response. Inhibitory effect of higher concentrations of sucrose on shoot organogenesis has been observed in cucumber [32]. However, in the present studies, glucose at its 4.0% concentration was found to be the most suitable carbon source as it produced maximum number (10.0) of elongated shoots. This concentration of glucose favoured elongation of shoots from all the cultured internodes. Glucose proved to be an efficient carbon source for tissue culture of *Ficus lyarata* [33], *Quercus suber* [34] carob [35] and beech [36] plants. Earlier reports demonstrate a variable effect of fructose as it was promotory for cultures of *Morus* [37] while inhibitory for *Syringa* [38]. The differential morphogenic response by the internodal explant to various sugars could be probably due to their differential role in vascular differentiation, differences in the endogenous content of reducing sugar in cultured tissue [39] and differential sensitivity of the tissues to the breakdown products such as furfural and hydroxyfurol formed during autoclaving.

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