

Full Length Research Paper

## Factors affecting *in vitro* plant regeneration from cotyledonary node explant of *Senna sophera* (L.) Roxb. – A highly medicinal legume

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An efficient *in vitro* regeneration protocol has been developed for a medicinal legume *Senna sophera* (L.) Roxb. using Cotyledonary Node (CN) explants. The plant exhibits high medicinal potential and is being used in several traditional and homeopathy system of medicine. The present study described an *in vitro* regeneration protocol, where different factors were optimized for maximum multiplication and propagation. The age of the explant, plant growth regulators, basal medium, pH of the medium and sucrose concentrations markedly influenced *in vitro* propagation of *S. sophera*. Among 14, 21 and 28 day-old CN explants, 21 day-old explants were found to be the most responsive. A maximum of  $19.50 \pm 0.51$  shoots/explant were produced from 21 day-old seedling explants, having an average shoot length of  $5.23 \pm 0.14$  cm in  $86.00 \pm 2.08\%$  cultures after 6 weeks of incubation on Murashige and Skoog (MS) medium supplemented with benzyl adenine (BA) ( $5.0 \mu\text{M}$ ) + naphthalene acetic acid (NAA) ( $1.0 \mu\text{M}$ ) and containing 3% sucrose with pH value adjusted at 5.8. The highest rooting frequency ( $96.00 \pm 2.08\%$ ) with maximum of  $7.63 \pm 0.23$  roots/shoot having an average root length of  $4.86 \pm 0.35$  cm was obtained on half-strength MS medium with  $1.0 \mu\text{M}$  indole-3-butyric acid (IBA) and solidified with 0.25% phytigel. The plantlets were acclimatized in sterile soilrite under controlled conditions, hardened and successfully transferred to soil in natural conditions with 90% survival rate. The regenerated plants showed no morphological variations in terms of leaf shape, flower shape, pod size, number of seeds etc., when compared with the naturally grown plants in the field.

**Key words:** *Senna sophera*, fabaceae, cotyledonary node, *in vitro* shoot regeneration, rooting, acclimatization.

### INTRODUCTION

*Senna sophera* (L.) Roxb. (*Cassia sophera* L., Fabaceae) is commonly known as 'kasondi' in Hindi. It is a diffuse, shrubby plant or under shrub about 0.7 to 3.0 m in height. The plant has been shown to exhibit anticancer activity and is reported to be extensively used in homeopathy (Pullaiah, 2006). The leaves possess purgative and anti-diuretic properties. It is used as an infusion or decoction

or as an expectorant for cough, cold, bronchitis, asthma, hiccups and jaundice and in sub-acute stages of gonorrhoea. Internally, it is reported to act as a febrifuge in rheumatic and inflammatory fevers and in skin diseases (Anonymous, 1992). The chemical analysis of the seeds revealed the presence of ascorbic acid, dihydroascorbic acid and  $\beta$ -sistosterol (Bilal et al., 2005). The powdered

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seeds mixed with honey are used in diabetes. In ethnobotanical literature, it is reported to be effective in the treatment of pityriasis and convulsions of children (Bilal et al., 2005). The methanol extract of the leaves of *C. sophera* can be used as a source of natural antioxidants with potential application to reduce oxidative stress with health benefits (Rahman et al., 2008). Conventionally, it is propagated through seeds; however, hard seed coat and seed dormancy prevent its germination in nature. Therefore, alternative techniques need to be developed for mass propagation and conservation of this valuable medicinal plant species. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases.

Advances in biotechnology, especially *in vitro* techniques provide an important tool for multiplication, conservation and propagation of several medicinal plants (Khallafalla and Daffalla, 2008; Shahzad et al., 2011; Li et al., 2012). Till date there is a single report on micro-propagation of *S. sophera* (Parveen and Shahzad, 2010). Thus, keeping in mind the use of *in vitro* approaches for the conservation of valuable medicinal plants; the present study was focused for *in vitro* propagation of *S. sophera* cotyledonary node explants.

## MATERIALS AND METHODS

### *In vitro* seed germination and explant collection

The certified seeds of *S. sophera* obtained from Prem Nursery and Seed Store, Dehradun (India), were used to raise aseptic seedlings. Seeds were washed thoroughly under running tap water for 30 min to remove adherent particles and kept in 1% (w/v) Bavistin (Cambendazim Powder), a broad spectrum fungicide, for 25 to 30 min. After that washed with 5% (v/v) Teepol, a liquid detergent, by continuous shaking for 15 min and then rinsed with sterile Double Distilled Water (DDW) for 3 to 4 times under the laminar flow hood followed by a short treatment of 70% (v/v) ethanol for 30 to 60 s and then surface sterilized with 0.1% (w/v) freshly prepared mercuric chloride (HgCl<sub>2</sub>) for 4 min.

Finally, the seeds were washed (5 to 6 times) with sterile DDW to remove the traces of sterilant. The sterilized seeds were inoculated in the culture flask (100 ml, Borosil) containing MS (Murashige and Skoog, 1962) basal medium or half-strength MS medium solidified with 0.8% (w/v) agar (Bacteriological grade, Hi-media) and containing 3% (w/v) sucrose and gibberellic acid (GA<sub>3</sub>) at various concentrations (0.5, 1.0 and 2.5 μM) or without GA<sub>3</sub>. Ten replicates were taken for each treatment and in each flask 10 seeds were inoculated.

### Culture media and conditions

Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Bacteriological grade, Hi-media) were used throughout the experiments. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl prior autoclaving at 121°C at 1.06 kg cm<sup>-2</sup> pressure for 20 min. All the cultures were maintained at 24 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by cool white fluorescent tubes (40W, Phillips, India) and with 50 to 60% relative humidity.

### Shoot induction, multiplication and maintenance

The MS medium supplemented with different cytokinins (BA, Kn and 2iP) at various concentrations (1.0, 2.5, 5.0, 7.5 and 10.0 μM) singly or in combination with different auxins (IAA, IBA and NAA) at various concentrations (0.5, 1.0 and 2.0 μM) were used for multiple shoot induction through CN explants excised from aseptic seedlings. For shoot multiplication and long-term establishment, the regenerating tissues were regularly sub cultured onto the fresh medium comprised of MS + BA (5.0 μM) + NAA (1.0 μM) every 6 weeks. The percentage of explants producing shoots, number of shoots/explant and shoot length were recorded after 6 weeks of culture.

### Effect of different media, pH and sucrose concentrations

Different factors like nutrients composition (B<sub>5</sub>, L<sub>2</sub>, MS and WPM medium), pH of the culture medium (5.0, 5.4, 5.8, 6.2 and 6.6) and sucrose concentration (1, 2, 3, 4 and 5%) have an influence on the induction and proliferation of multiple shoots. Thus, in the present investigation, all these three factors were standardized for the optimum regeneration of shoots via CN explant in *S. sophera*.

### *In vitro* rooting in microshoots

Regenerated shoots of about 4 to 5 cm length were excised and transferred to the rooting media comprised of MS basal and half-strength MS medium solidified with 0.25% phytigel and supplemented with different auxins like IAA, IBA and NAA (0.5, 1.0 and 2.5 μM). Data were recorded for rooting percentage, mean number of roots/shoot and root length after 4 weeks of transfer onto the rooting medium.

### Hardening and acclimatization

Plantlets with well-developed root and shoot system were acclimatized as per the standard procedure adopted by Parveen and Shahzad (2010).

### Data collection and statistical analysis

The data for percentage regeneration, number of shoots per explants and shoot length were recorded after 6 weeks and for rooting experiment after 4 weeks. All the experiments were conducted with a minimum of ten replicates per treatment and repeated three times. The data were analyzed statistically using SPSS ver.16 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test (DMRT) at  $P \leq 0.05$ ; the results are expressed as a mean ± SE of three repeated experiments.

## RESULTS AND DISCUSSION

About 99.3% seeds germinated on half-strength MS medium containing 1.0 μM GA<sub>3</sub> (data not shown). Cotyledonary nodes (CN) excised from 14, 21 and 28 day-old aseptic seedlings were used for the selection of the most responsive age of the explant and establishment of *in vitro* cultures.

**Table 1.** Effect of age of cotyledonary nodes on direct shoot regeneration in *S. sophera* cultured on MS medium containing different concentrations of BA.

BA ( $\mu\text{M}$ )	14 day-old explants		21 day-old explants		28 day-old explants	
	Regeneration (%)	Mean number of shoots per explant	Regeneration (%)	Mean number of shoots per explant	Regeneration (%)	Mean number of shoots per explant
-	00.00 $\pm$ 0.00 <sup>e</sup>	0.00 $\pm$ 0.00 <sup>e</sup>	0.00 $\pm$ 0.00 <sup>e</sup>	0.00 $\pm$ 0.00 <sup>d</sup>	0.00 $\pm$ 0.00 <sup>e</sup>	0.00 $\pm$ 0.00 <sup>e</sup>
1.0	45.66 $\pm$ 3.48 <sup>d</sup>	3.30 $\pm$ 0.20 <sup>c</sup>	55.66 $\pm$ 2.33 <sup>d</sup>	4.66 $\pm$ 0.20 <sup>c</sup>	35.00 $\pm$ 2.88 <sup>d</sup>	3.20 $\pm$ 0.17 <sup>d</sup>
2.5	54.33 $\pm$ 2.33 <sup>b</sup>	5.23 $\pm$ 0.14 <sup>b</sup>	67.33 $\pm$ 2.33 <sup>c</sup>	8.83 $\pm$ 0.32 <sup>b</sup>	48.33 $\pm$ 2.90 <sup>bc</sup>	3.96 $\pm$ 0.14 <sup>bc</sup>
5.0	62.33 $\pm$ 1.45 <sup>a</sup>	9.06 $\pm$ 0.23 <sup>a</sup>	78.66 $\pm$ 2.33 <sup>a</sup>	12.43 $\pm$ 0.29 <sup>a</sup>	56.33 $\pm$ 2.33 <sup>a</sup>	6.56 $\pm$ 0.23 <sup>a</sup>
7.5	52.66 $\pm$ 1.76 <sup>bc</sup>	5.80 $\pm$ 0.20 <sup>b</sup>	72.66 $\pm$ 1.45 <sup>b</sup>	9.76 $\pm$ 0.43 <sup>b</sup>	50.00 $\pm$ 2.86 <sup>ab</sup>	4.56 $\pm$ 0.23 <sup>b</sup>
10.0	47.33 $\pm$ 2.02 <sup>cd</sup>	3.93 $\pm$ 0.23 <sup>d</sup>	64.00 $\pm$ 3.05 <sup>c</sup>	5.90 $\pm$ 0.20 <sup>c</sup>	42.33 $\pm$ 1.45 <sup>c</sup>	3.66 $\pm$ 0.26 <sup>cd</sup>

-Data recorded after 6 weeks.

-Values represent Mean  $\pm$  SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ( $P \leq 0.05$ ) using Duncan's multiple range test (DMRT).

### Effect of explant age on multiple shoot regeneration

The age of the explant played an important role on the onset of morphogenesis in *in vitro* cultures as the explants excised from seedlings or younger plants have more regeneration capacity than explants collected from mature plants (Distanbanjong and Geneve, 1997). Therefore, to evaluate the efficiency of shoot regeneration, cotyledonary node explants excised from seedlings of different age group (14, 21 and 28 day-old) were cultured on MS medium containing various concentrations of BA. MS medium without BA served as control treatment. All the three different aged explants failed to respond on hormone free MS medium and died within 2 weeks of inoculation. Among different aged explants, 21 day-old seedling explants produced a maximum of  $12.43 \pm 0.29$  shoots/explant on MS + BA (5.0  $\mu\text{M}$ ) after 6 weeks of inoculation in  $78.66 \pm 2.33\%$  cultures. On this medium, induction of shoot buds started after 9 to 10 days of incubation. On the same composition of medium, the explants isolated from 14 day-old seedlings produced  $9.06 \pm 0.23$  shoots/explant with  $62.33 \pm 1.45\%$  response, while, the explants obtained from 28 days old seedlings produced only  $6.56 \pm 0.23$  shoots/explant in  $56.33 \pm 2.33\%$  cultures after 6 weeks of inoculation (Table 1). Similarly, Jeyakumar and Jayabalan (2002) reported better regeneration efficiency of CN explants collected from 20 days old aseptic seedlings of *Psoralea corylifolia*. Explants containing axillary/apical buds have quiescent or active meristems depending upon the physiological stage of the plant. These buds have the potential to develop into complete plantlets.

But, in nature, these buds remain dormant for a specific period of time depending on the growth pattern of the plant (Rani and Rana, 2010). Likewise, there are many reports available, where the age of the explant affected the rate of shoot regeneration (Gitonga et al., 2010; Devi et al., 2011; Mohebodini et al., 2011).

### Effect of cytokinins on multiple shoot regeneration

The best responsive CN explants excised from 21 day-

old seedlings were cultured on MS medium containing three different cytokinins (BA, Kn and 2iP) for multiple shoot regeneration through direct organogenesis. MS medium without cytokinins (control) did not stimulate any morphogenic response and the explants died within two weeks of culture. However, supplementation of various cytokinins at different concentrations facilitated induction of multiple shoots from CN explants. Among all the three cytokinins tested, BA was proved to be the best and the number of shoots increased with an increase in the concentration up to 5.0  $\mu\text{M}$ , beyond that a decline in percent response as well as number of shoots was recorded (Table 2). Enlargement and swelling of the explants was observed within 4 to 5 days of incubation followed by emergence of green protuberances from the swelled region after 9 to 10 days. At 5.0  $\mu\text{M}$  BA, a maximum of  $12.43 \pm 0.29$  shoots/explant attaining an average shoot length of  $4.23 \pm 0.26$  cm was obtained in a maximum of  $78.66 \pm 2.33\%$  cultures after 6 weeks of inoculation (Figure 1A). Higher concentrations of BA (7.5 and 10.0  $\mu\text{M}$ ) found to be inhibitory for shoot regeneration because of huge basal callusing which hampered the growth and development of new shoots. At 10.0  $\mu\text{M}$  BA, a decrease in the percent response ( $64.00 \pm 3.05\%$ ) was noticed and the number of shoots was reduced to  $5.90 \pm 0.20$  shoots/explant after 6 weeks of culture because callus formation acts as a mechanical barrier to nutrient and water uptake as suggested by De Klerk (2002). These findings substantiate with the earlier reports of *Ocimum* spp. (Pattnaik and Chand, 1996), *Gymnema sylvestri* (Reddy et al., 1998); *Entada phaseoloids* (Rao and Vishnupriya, 2002) and *Saussurea obvallata* (Joshi and Dhar, 2003). To check the growth of basal callus, the cultures were frequently transferred onto the fresh medium of same composition after removing the callus mass after every 2 weeks.

In contrast to BA, Kn and 2iP were effective at higher concentration that is, 7.5  $\mu\text{M}$  where induction of shoot buds began after 14 days of inoculation in both Kn and 2iP supplemented media. Shoots were not as healthy as that of BA supplemented media and were having small

**Table 2.** Effect of different cytokinins on direct shoot regeneration from cotyledonary nodes of *S. sophera*.

PGR ( $\mu\text{M}$ )			Regeneration (%)	Mean number of shoots/explant	Mean shoot length (cm)
BA	Kn	2iP			
1.0	-	-	55.66 $\pm$ 2.33 <sup>ef</sup>	4.66 $\pm$ 0.20 <sup>f</sup>	3.00 $\pm$ 0.11 <sup>cde</sup>
2.5	-	-	67.33 $\pm$ 2.33 <sup>bc</sup>	8.83 $\pm$ 1.32 <sup>c</sup>	3.46 $\pm$ 0.14 <sup>bc</sup>
5.0	-	-	78.66 $\pm$ 2.33 <sup>a</sup>	12.43 $\pm$ 0.29 <sup>a</sup>	4.23 $\pm$ 0.26 <sup>a</sup>
7.5	-	-	72.66 $\pm$ 1.45 <sup>b</sup>	9.76 $\pm$ 0.43 <sup>b</sup>	3.90 $\pm$ 0.20 <sup>ab</sup>
10.0	-	-	64.00 $\pm$ 3.05 <sup>cd</sup>	5.90 $\pm$ 0.20 <sup>de</sup>	3.26 $\pm$ 0.14 <sup>cd</sup>
-	1.0	-	00.00 $\pm$ 0.00 <sup>l</sup>	0.00 $\pm$ 0.00 <sup>j</sup>	0.00 $\pm$ 0.00 <sup>g</sup>
-	2.5	-	40.66 $\pm$ 2.33 <sup>ij</sup>	3.63 $\pm$ 0.23 <sup>g</sup>	2.60 $\pm$ 0.20 <sup>ef</sup>
-	5.0	-	44.33 $\pm$ 2.96 <sup>hi</sup>	5.63 $\pm$ 0.23 <sup>e</sup>	3.00 $\pm$ 0.11 <sup>cde</sup>
-	7.5	-	60.33 $\pm$ 1.45 <sup>de</sup>	6.56 $\pm$ 0.34 <sup>d</sup>	3.56 $\pm$ 0.29 <sup>bc</sup>
-	10.0	-	52.33 $\pm$ 1.45 <sup>fg</sup>	4.43 $\pm$ 0.23 <sup>f</sup>	3.23 $\pm$ 0.14 <sup>cd</sup>
-	-	1.0	00.00 $\pm$ 0.00 <sup>l</sup>	00.00 $\pm$ 0.00 <sup>j</sup>	00.00 $\pm$ 0.00 <sup>f</sup>
-	-	2.5	33.00 $\pm$ 2.08 <sup>k</sup>	2.06 $\pm$ 0.23 <sup>i</sup>	2.43 $\pm$ 0.23 <sup>f</sup>
-	-	5.0	36.33 $\pm$ 2.02 <sup>jk</sup>	2.90 $\pm$ 0.20 <sup>h</sup>	2.76 $\pm$ 0.14 <sup>def</sup>
-	-	7.5	47.66 $\pm$ 1.45 <sup>gh</sup>	4.23 $\pm$ 0.14 <sup>fg</sup>	3.23 $\pm$ 0.14 <sup>cd</sup>
-	-	10.0	37.66 $\pm$ 1.45 <sup>k</sup>	2.53 $\pm$ 0.20 <sup>hi</sup>	2.73 $\pm$ 0.14 <sup>def</sup>

-Data recorded after 6 weeks.

-Values represent Mean  $\pm$  SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ( $P \leq 0.05$ ) using Duncan's multiple range test (DMRT).

leaves with thin stem. The lower concentration (1.0  $\mu\text{M}$ ) of both Kn and 2iP failed to provide any response. However, as the concentration of cytokinins was further increased a linear increase in the number of shoots was observed up to an optimal level (7.5  $\mu\text{M}$ ). The MS medium containing 7.5  $\mu\text{M}$  of Kn produced an average of 6.56  $\pm$  0.34 shoots/explant with a regeneration potential of 60.33  $\pm$  1.45%. But, 2iP at the same concentration yielded only 4.23  $\pm$  0.14 shoots/explant in 47.66  $\pm$  1.45% cultures after 6 weeks of inoculation. Beyond the optimal concentration (7.5  $\mu\text{M}$ ), a decrease in the response as well as number of shoots was recorded due to profuse basal callusing (Table 2). Similarly, the superiority of BA on bud break and multiple shoot production has also been reported earlier in several medicinal and aromatic plants including *Mucuna pruriense* (Sathyanarayana et al., 2008); *Clitoria ternatea* (Singh and Tiwari, 2010); *Justicia gendarussa* (Thomas and Yoichiro, 2010) and *Veronica anagallis-aquatica* (Shahzad et al., 2011).

### Effect of cytokinin-auxin combinations on multiple shoot regeneration

In general, higher concentration of cytokinin and lower concentration of auxin are required in a medium to promote multiplication and proliferation of shoots (Kohlenbach, 1997; Beena et al., 2003). In the present study, the optimal concentrations that is, 5.0  $\mu\text{M}$  of BA was also tested with three auxins to evaluate the synergistic effect of cytokinin-auxin combinations. Among various combinations, BA + NAA combination treatments provided best

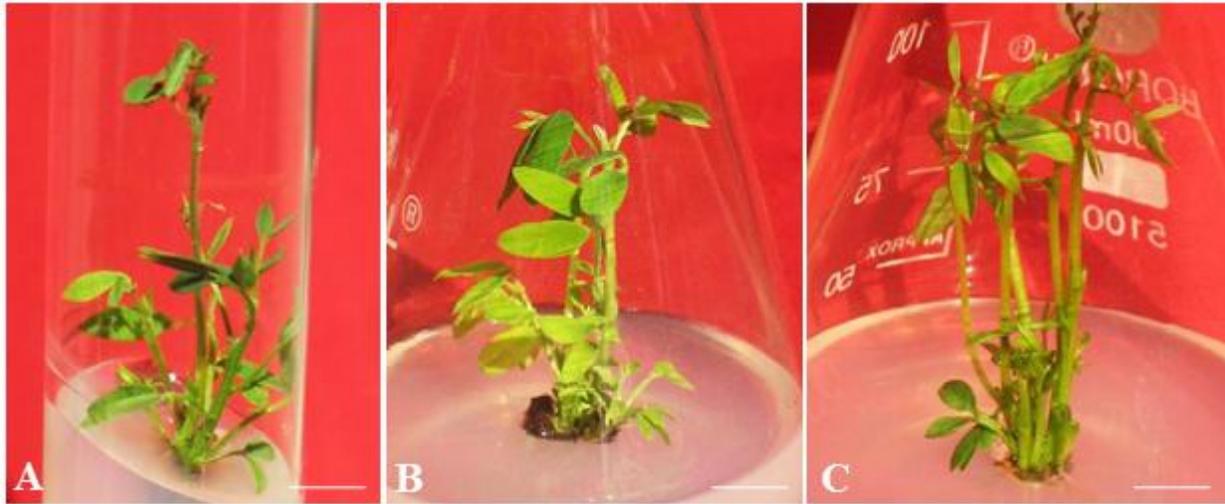
response (Figure 1B). Induction of shoot buds took place within 8 days of incubation with slight swelling of the explant on MS + BA (5.0  $\mu\text{M}$ ) + NAA (1.0  $\mu\text{M}$ ) and yielded a maximum 19.50  $\pm$  0.51 shoots/explant in 86.00  $\pm$  2.08% cultures with 5.23  $\pm$  0.14 cm shoot length after 6 weeks of inoculation (Table 3 and Figure 1C).

The synergistic effects of BA-NAA combination have also been reported in other plant species such as *Rauvolfia serpentina* (Baksha et al., 2007), *Baliospermum montanum* (George et al., 2008) and *Boerhaavia diffusa* (Biswas et al., 2009). The number of shoots per explant was found to be more in this study compared to our previous report (Parveen and Shahzad, 2010) on the same plant, where thidiazuron (TDZ) was used. Though, the addition of higher concentration (2.0  $\mu\text{M}$ ) of NAA resulted in callus formation and thereby decline in mean number of shoots/explant (13.93  $\pm$  0.23) as well as in percent response (80.33  $\pm$  1.45%).

In the other combination treatments, BA + IBA combination was better than BA + IAA. The medium comprised of MS medium with optimal concentration of BA (5.0  $\mu\text{M}$ ) and IBA (1.0  $\mu\text{M}$ ) exhibited 83.00  $\pm$  1.15% response producing an average of 14.83  $\pm$  0.37 shoots/explant, while IAA at the same concentration provided 13.56  $\pm$  0.23 shoots/explant with 77.33  $\pm$  1.45% response after 6 weeks of culture (Table 3).

### Effect of different media

Some species give similar response in all the media while others show preference for a specific medium for the esta-



**Figure 1.** Multiple shoot regeneration in *S. sophora* through cotyledonary node explants on MS medium supplemented with various PGRs. A) Induction of multiple shoots on MS + BA (5.0  $\mu$ M), bar = 0.73 cm. B) Multiple shoots development on MS + BA (5.0  $\mu$ M) + NAA (0.5  $\mu$ M), bar = 1.0 cm. C) Multiplication and proliferation of shoots on MS + BA (5.0  $\mu$ M) + NAA (1.0  $\mu$ M), bar = 0.92 cm.

**Table 3.** Effect of optimal concentration of BA with different auxins on direct shoot regeneration from cotyledonary nodes of *S. sophora*.

BA	PGR ( $\mu$ M)			Regeneration (%)	Mean number of shoots/explant	Mean shoot length (cm)
	NAA	IBA	IAA			
5.0	0.5	-	-	82.33 $\pm$ 1.45 <sup>abc</sup>	15.26 $\pm$ 0.17 <sup>b</sup>	4.76 $\pm$ 0.14 <sup>ab</sup>
5.0	1.0	-	-	86.00 $\pm$ 2.08 <sup>a</sup>	19.50 $\pm$ 0.51 <sup>a</sup>	5.23 $\pm$ 0.14 <sup>a</sup>
5.0	2.0	-	-	80.33 $\pm$ 1.45 <sup>bcd</sup>	13.93 $\pm$ 0.23 <sup>c</sup>	4.26 $\pm$ 0.14 <sup>c</sup>
5.0	-	0.5	-	79.33 $\pm$ 1.76 <sup>bcd</sup>	12.90 $\pm$ 0.20 <sup>de</sup>	4.23 $\pm$ 0.14 <sup>c</sup>
5.0	-	1.0	-	83.00 $\pm$ 1.15 <sup>ab</sup>	14.83 $\pm$ 0.37 <sup>b</sup>	4.96 $\pm$ 0.14 <sup>ab</sup>
5.0	-	2.0	-	77.66 $\pm$ 1.45 <sup>cd</sup>	12.53 $\pm$ 0.20 <sup>e</sup>	4.10 $\pm$ 0.20 <sup>c</sup>
5.0	-	-	0.5	75.66 $\pm$ 1.76 <sup>d</sup>	12.23 $\pm$ 0.14 <sup>ef</sup>	4.00 $\pm$ 0.11 <sup>c</sup>
5.0	-	-	1.0	77.33 $\pm$ 1.45 <sup>cd</sup>	13.56 $\pm$ 0.23 <sup>cd</sup>	4.50 $\pm$ 0.17 <sup>bc</sup>
5.0	-	-	2.0	75.00 $\pm$ 1.73 <sup>d</sup>	11.46 $\pm$ 0.31 <sup>f</sup>	4.03 $\pm$ 0.14 <sup>c</sup>

-Data recorded after 6 weeks.

-Values represent Mean  $\pm$  SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ( $P \leq 0.05$ ) using Duncan's multiple range test (DMRT).

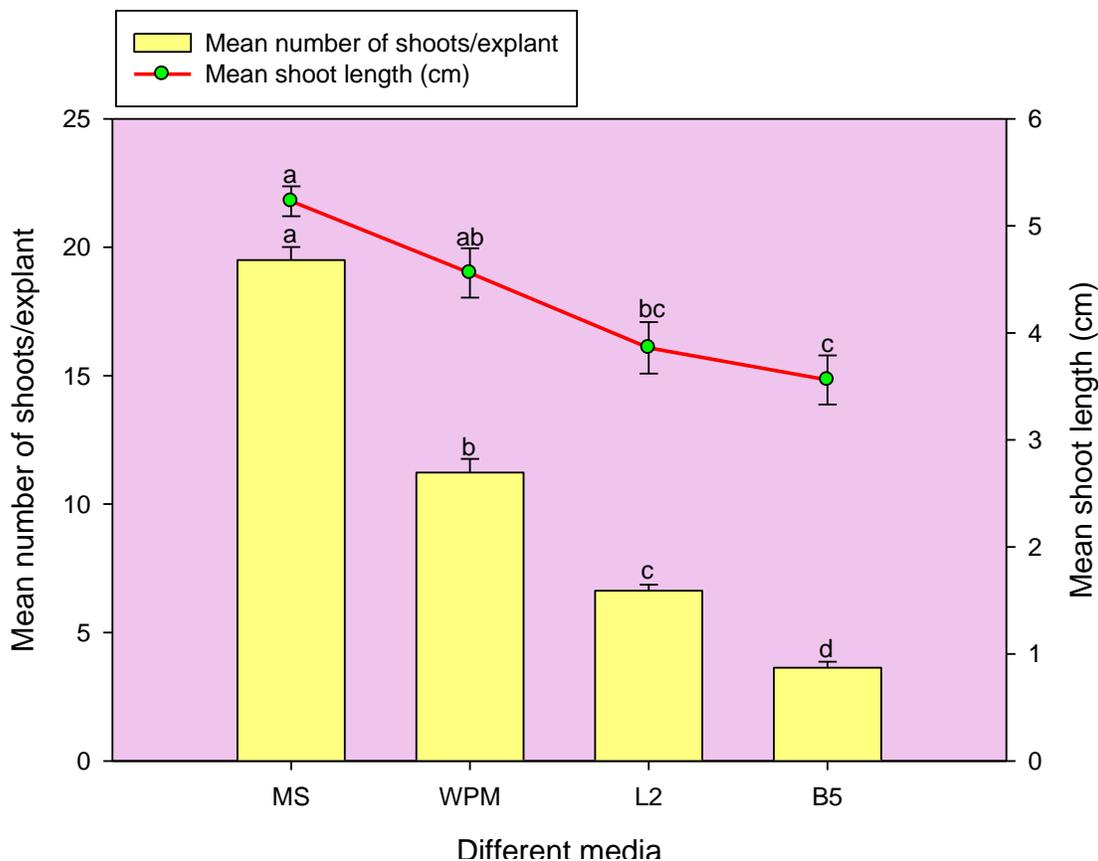
blishment and proliferation of cultures (McCown and Sellmer, 1987). Amongst all the four media tested, MS medium was found to be the most suitable medium for multiple shoots regeneration from CN explant and yielded maximum 19.50  $\pm$  0.51 shoots/explant with highest shoot length of 5.23  $\pm$  0.14 cm on the optimized medium that is, MS + BA (5.0  $\mu$ M) + NAA (1.0  $\mu$ M) after 6 weeks of culture (Figure 2).

On MS medium shoot buds were induced within 8 days of culture. Multiple shoot buds were differentiated on WPM, L<sub>2</sub> and B<sub>5</sub> media but showed limited growth and development even if they were maintained for longer period in culture. MS medium has also been found more effective than other media in various medicinal plants (Wondyifraw and Surawit, 2004; Wang et al., 2008).

However, contrary to our results, WPM was found to be the best medium for shoot multiplication compared to MS and AN (Anderson's rhododendron medium) medium in *Vaccinium arctostaphylos* by Cüce et al. (2013).

### Effect of pH

The effect of different pH tested in MS medium comprised of optimized concentrations of BA (5.0  $\mu$ M) and NAA (1.0  $\mu$ M) on shoot regeneration from CN explants. The ideal value of pH for the maximum regeneration proved to be 5.8 where a maximum of 19.50  $\pm$  0.51 shoots/CN explant was obtained after 6 weeks of culture. pH values below the optimal level resulted in the production of loose or watery medium which was slight acidic



**Figure 2.** Effect of different culture media supplemented with BA (5.0  $\mu$ M) and NAA (1.0  $\mu$ M) on shoot regeneration from cotyledonary nodes of *C. sophora* after 6 weeks of culture.

in nature and declined the number of shoots to  $10.63 \pm 0.29$  and  $6.83 \pm 0.27$  shoots/explant at pH 5.4 and 5.0, respectively. High pH values produced alkaline medium which affected the regeneration potential of the explants and thus, reduced the number of shoots to  $8.43 \pm 0.29$  and  $5.50 \pm 0.17$  shoots/explant at pH 6.2 and 6.6, respectively (Figure 3). It is known that the medium pH has effects not only on the uptake of nutrients but also on chemical reactions especially those catalyzed by enzymes (Thorpe et al., 1991).

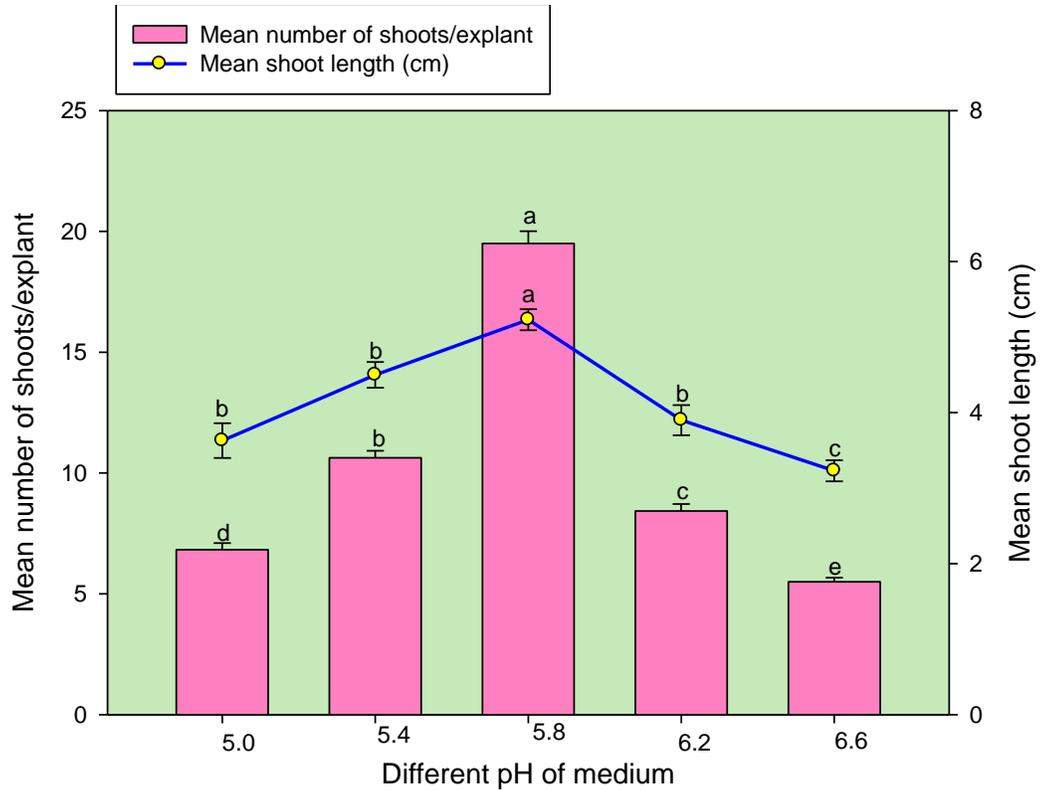
Our findings substantiate with earlier reports on other plants where pH 5.8 was found to be the optimum value (Nair and Seenii, 2003). Hydrogen ion concentration (pH) of the culture medium is one of the important factors of physico-chemical environment during development of plant tissues under *in vitro* conditions (Williams et al., 1990). The optimal pH of the medium varies according to the different phases of morphogenesis namely; establishment of cultures, shoot proliferation and induction of roots (Saborio et al., 1997; Ostrolucká et al., 2004).

#### Effect of sucrose concentrations

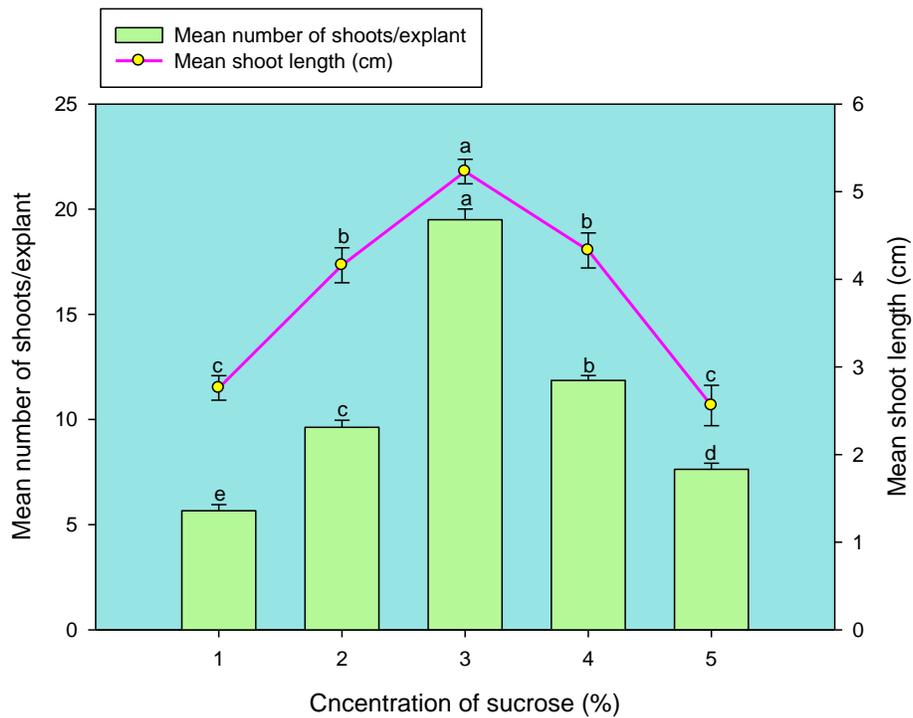
Plant cell, tissue or organ culture normally require the incorporation of a carbon or energy source to the culture

medium (Karhu, 1997) and sucrose has been used as the major carbon source in plant tissue culture (Fuentes et al., 2000). The best response was exhibited by the explants on medium comprising 3% sucrose, producing a maximum of  $19.50 \pm 0.51$  shoots/explant attaining the highest shoot length of  $5.23 \pm 0.14$  cm on the optimized medium that is, MS + BA (5.0  $\mu$ M) + NAA (1.0  $\mu$ M) after 6 weeks of culture. While, below this optimal concentration of sugar the explants showed less response and consequently the number of shoots declined to  $9.63 \pm 0.34$  and  $5.66 \pm 0.29$  shoots/explant at 2 and 1% sucrose, respectively. Higher concentrations of sucrose also reduced the regeneration efficacy of the explant and number of shoots was reduced to  $11.86 \pm 0.23$  and  $7.63 \pm 0.29$  at 4 and 5% of sucrose, respectively (Figure 4). Similar to our results, Anitha and Pullaiah (2002) also obtained optimal regeneration at 3% sucrose in comparison to other concentrations of sucrose. However in *Eucomis autumnalis*, maximum regeneration was obtained at 4% sucrose and the lower concentrations decreased the regeneration rate (Taylor and van Staden, 2001); similarly, in *Amygdalus communis* shoot multiplication was obtained only at 5 and 6% sucrose (Gürel and Gülsen, 1998) which is contrary to our results.

On the other hand, majority of micropropagation proto-



**Figure 3.** Effect of different pH values on shoot regeneration from cotyledonary nodes of *C. sophera* on MS medium containing optimal concentration of BA (5.0  $\mu$ M) and NAA (1.0  $\mu$ M) after 6 weeks of culture.



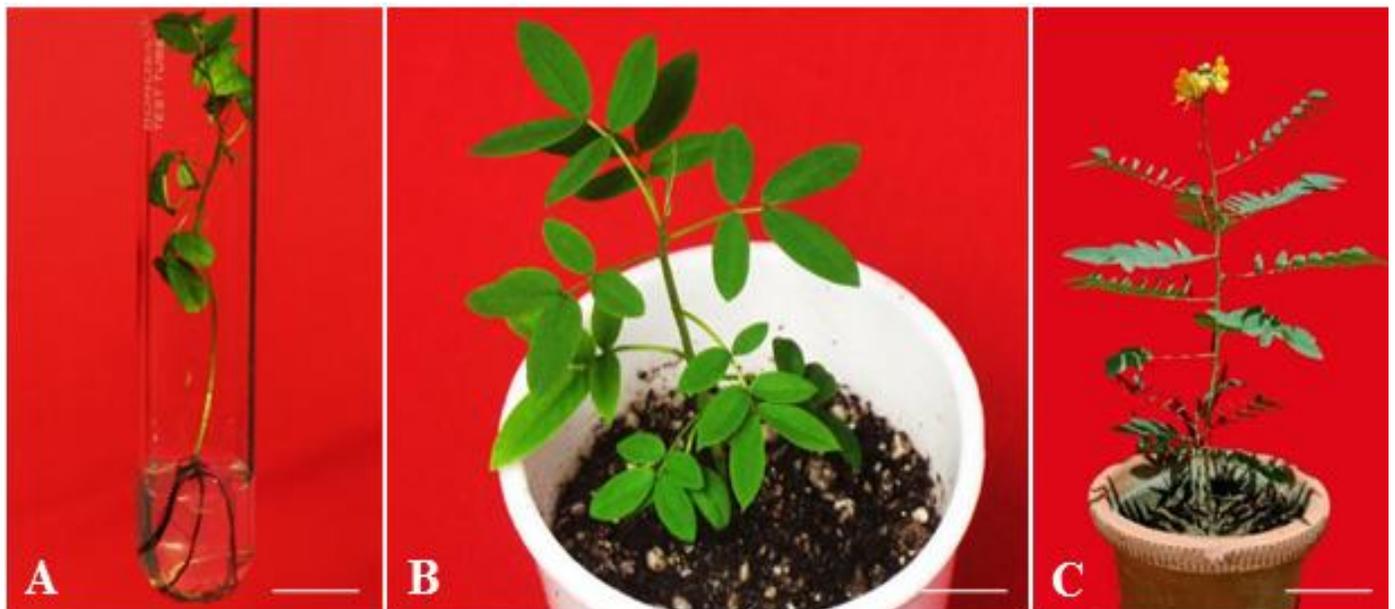
**Figure 4.** Effect of different concentrations of sucrose on shoot regeneration from cotyledonary nodes of *C. sophera* on optimal medium comprised of MS + BA (5.0  $\mu$ M) + NAA (1.0  $\mu$ M) after 6 weeks of culture.

**Table 4.** Effect of different auxins on *in vitro* root induction in microshoots of *S. sophera* in phytigel solidified medium.

Treatment ( $\mu\text{M}$ )	Response (%)	Mean number of roots/shoot	Mean root length (cm)
MS	47.00 $\pm$ 1.73 <sup>h</sup>	3.70 $\pm$ 0.58 <sup>e</sup>	1.63 $\pm$ 0.23 <sup>f</sup>
½ MS	66.66 $\pm$ 1.76 <sup>g</sup>	4.23 $\pm$ 0.14 <sup>de</sup>	2.23 $\pm$ 0.14 <sup>ef</sup>
½ MS + IBA (0.5)	85.00 $\pm$ 1.73 <sup>b</sup>	4.90 $\pm$ 0.20 <sup>d</sup>	4.86 $\pm$ 0.35 <sup>a</sup>
½ MS + IBA (1.0)	96.00 $\pm$ 2.08 <sup>a</sup>	7.63 $\pm$ 0.23 <sup>a</sup>	4.66 $\pm$ 0.35 <sup>a</sup>
½ MS + IBA (2.5)	82.33 $\pm$ 1.45 <sup>bc</sup>	6.90 $\pm$ 0.20 <sup>ab</sup>	3.56 $\pm$ 0.23 <sup>bc</sup>
½ MS + NAA (0.5)	75.33 $\pm$ 1.45 <sup>de</sup>	4.50 $\pm$ 0.28 <sup>de</sup>	3.10 $\pm$ 0.20 <sup>cd</sup>
½ MS + NAA (1.0)	79.66 $\pm$ 1.45 <sup>cd</sup>	6.60 $\pm$ 0.23 <sup>bc</sup>	3.83 $\pm$ 0.20 <sup>b</sup>
½ MS + NAA (2.5)	69.00 $\pm$ 2.08 <sup>fg</sup>	4.16 $\pm$ 0.20 <sup>de</sup>	2.50 $\pm$ 0.17 <sup>de</sup>
½ MS + IAA (0.5)	67.66 $\pm$ 1.45 <sup>fg</sup>	3.93 $\pm$ 0.29 <sup>e</sup>	2.43 $\pm$ 0.17 <sup>de</sup>
½ MS + IAA (1.0)	72.33 $\pm$ 1.45 <sup>ef</sup>	5.96 $\pm$ 0.14 <sup>c</sup>	3.23 $\pm$ 0.14 <sup>b<sup>c</sup></sup>
½ MS + IAA (2.5)	65.33 $\pm$ 1.45 <sup>g</sup>	3.90 $\pm$ 0.20 <sup>e</sup>	2.23 $\pm$ 0.14 <sup>ef</sup>

-Data recorded after 4 weeks.

-Values represent Mean  $\pm$  SE of three repeated experiments with 10 replicates each. Means followed by the same letter within columns are not significantly different ( $P \leq 0.05$ ) using Duncan's multiple range test (DMRT).



**Figure 5.** *In vitro* rooting and acclimatization of regenerated plantlets of *S. sophera*. A) *In vitro* root induction in phytigel solidified medium containing ½ MS + IBA (1.0  $\mu\text{M}$ ), bar = 1.78 cm. B) An acclimatized plantlet in soilrite, bar = 1.30 cm. C) *In vitro* regenerated plant of *S. sophera* in soil showing development of inflorescence, bar = 8.03 cm.

cols generally utilize 3% sucrose as per recommendation of Murashige and Skoog (1962). Exogenous supply of sugar increases starch and sucrose reserves in micropropagated plants and could favour *ex vitro* acclimatization and speed up physiological adaptations (Pospíšilová et al., 1999).

### ***In vitro* rooting**

The development of healthy root system is required for the successful establishment of regenerated shoots in the external environment. Hence, appropriate sized (4 to 5

cm) microshoots of *S. sophera* were excised from the regenerating cultures and transferred to different rooting media for the induction of *in vitro* roots. Induction of roots from the cut ends of the shoots took place within 10 to 12 days and thin, dark brown to black roots were produced in all the treatments. The best rooting (96.00  $\pm$  2.08%) was achieved on half-strength MS medium solidified with 0.25% phytigel and containing 1.0  $\mu\text{M}$  IBA, wherein roots were induced after 7 to 8 days and an average of 7.63  $\pm$  0.23 roots/shoot having average root length of 4.66  $\pm$  0.35 cm were produced after 4 weeks of transfer (Table 4 and Figure 5A). Higher concentration (2.5  $\mu\text{M}$ ) of IBA re-

reduced the rooting potential and resulted in the formation of short roots. NAA and IAA proved to be less effective than IBA for root induction. Our results are in accordance with various findings where IBA proved to be more efficient for rooting than other auxins in number of plants such as *Cunila galoides* (Fracaro and Echeverrigaray, 2001).

### Hardening and acclimatization

Hardening and acclimatization of plantlets is essential for the survival of regenerated plantlets in the external environment. Regenerated plantlets with 4 to 5 fully expanded leaves and well-developed root system were hardened off under aseptic conditions in sterile soilrite. Hardening of plantlets was done by the procedure described in the materials and methods. Almost 90% plantlets survived in the field conditions after successful acclimatization and exhibited normal growth and development pattern with no detectable morphological variations when compared with the *in vivo* grown plants (Figure 5B and C).

### Conclusion

The comprehensive protocol described here is efficient, reproducible and could be used in future for mass propagation and biotechnological programs for the genetic transformation or manipulation of highly medicinal legume *S. sophora*.

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