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In vitro Shoot Regeneration of *Stereospermum suaveolens* DC. using Cotyledonary Node and Nodal Explants

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Abstract

The cotyledonary node and nodal explants *Stereospermum suaveolens* DC. were cultured on Woody Plant medium (WPM) supplemented with individual BAP 2 - 25 μM), Kn (2 - 25 μM) and TDZ (0.1 - 2 μM. Observations revealed that in both cotyledonary node and nodal explants**,** lower concentrations of cytokinins (BAP/Kn/TDZ) were effective in inducing proliferation response. The axillary bud of both the explants proliferated into single shoots which were further subcultured on fresh medium with respective concentrations for shoot multiplication. It was observed that multiple shoots developed only in presence of Kn (8 μM) in both the explants but the shoots developed from nodal explants were strong and healthy with average shoot number reaching to 3.8 ± 0.6 and length 5.4 ± 0.4 cm by the end of third passage. Rooting (100%) was achieved in microshoots when transferred to half strength WPM liquid medium supplemented with IBA (2 μM). The plantlets were transferred to different substrates for acclimatization.

Introduction

Forest trees have been exploited over the years as they are the renewable sources of food, fodder, fuel wood, timber and medicinal properties. Due to rapid growth of population there has been a tremendous reduction in forest cover from the earth (Giri et al. 2004) and number of plants in the wild has progressively declined pushing them into endangered category. Hence, steps have to be taken towards conservation and sustainable utilization of important forest trees. To propagate trees on large scale, vegetative methods become difficult and time consuming, an alternative method like *in vitro* propagation could be the solution

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for this and several other woody species (Thorpe et al. 1991, Ahuja 1993, Vinocur et al. 2000). *In vitro* techniques can produce clonal planting stock for afforestation, woody biomass production and conservation of elite and rare germplasm (Rout et al. 2008).

Stereospermum suaveolens DC. is a valuable tree species belonging to family Bignoniaceae (Anon. 1998). Roots of the plant are the source of active components, used in the preparation of ayurvedic formulations like Dashmoolarisht and Chywanprash where the whole plant is uprooted resulting in total destruction of the species (Yashoda et al. 2004). Conventionally the plant is propagated through seeds, however it is a tedious process as the seeds are winged creating difficulty in its collection and they also have a poor germination rate (Baul et al. 2009). Hence there is a need to conserve this important tree species which can be achieved through *in vitro* techniques. There are many reports documenting successful establishment of cultures utilizing cotyledonary nodes in tree species like *Sterculia urens* (Purohit and Dave 1996), *Murraya koenigii* (Bhuyan et al. 1997), *Dalbergia sissoo* (Pradhan et al. 1998), *Quercus floribunda* (Purohit et al. 2002), *Cassia sophera* (Parveen and Shahzad 2010). Whereas plants like *Psidium gujava* L. (Amin and Jaiswal 1987), *Sterculia urens* Roxb. (Devi et al. 2011), *Balanites aegyptiaca* L. (Siddique and Anis 2009), *Vitex negundo* L. (Ahmad and Anis 2007) have been regenerated utilizing nodal explants. Therefore, the present work was aimed to study the *in vitro* regeneration potential of *S. suaveolens* from cotyledonary node and nodal explants in WPM medium fortified with different individual cytokinins.

Materials and Methods

The present year seeds of *S. suaveolens* DC. were collected from Rajpila Forest Division and germinated in cocopeat for obtaining a large number of seedling explants (Trivedi and Joshi 2014). Healthy seeds were presoaked overnight, next day kept under running water and then washed with Teepol for 5-10 min. A treatment of bavistin (200 mg/l) was given for 2 min and thereafter the seeds were surface sterilized with $HgCl_2$ (0.1%) for 2 min. They were thoroughly washed three times with sterile distiled water and germinated singly in each well of root trainer which was filled with sterile cocopeat. The root trainers were kept in culture room at 25 ± 2°C.

Twenty day old seedlings were selected and kept under running water for 2 hrs, washed with Teepol for 5 - 10 min. They were treated with PVP (100 mg/l) for 3 min, followed by bavistin (200 mg/l) treatment for 3 min. and then surface sterilized with $HgCl_2$ (0.1%) for 3 min, finally rinsed 3 - 4 times with sterile distil water. Cotyledonary node (1 - 1.5 cm) was excised aseptically from these

seedlings. Nodal segments (1 - 2 cm) collected from 1 - 2 year old plants were treated similarly with PVP (100 mg/l) for 3 min, followed by bavistin (200 mg/l) treated for 5 min, then by an additional treatment of streptomycin (100 mg/l) for 5 min, lastly surface sterilization with $HgCl_2$ (0.1%) for 5 min was given and finally rinsed (3 - 4 times) with sterile distiled water.

Cotyledonary nodes and nodal explants were placed on WPM medium supplemented individually with BAP/Kn (2, 4, 8, 16, 20, 25 μM) and TDZ (0.1, 0.2, 0.25, 0.5, 1, 2 μM). One set of both the explants were cultured on basal medium which served as a control. The pH of all the media was adjusted to 5.8 and solidified with 0.8% agar before autoclaving and sterilization was done at 121°C with 15 psi for 25 min. All the cultures were incubated at 25 ± 2°C and the data for shoot proliferation were recorded after four weeks in all the explants.

The *in vitro* shoots were cut into single nodal segments and transferred to fresh medium of the same concentration for multiplication after four weeks interval. The data for per cent response, number of shoots, shoot length were recorded after eight weeks.

The elongated *in vitro* shoots (4 - 5 cms) were transferred to induce roots in basal (liquid and static) full and half strength WPM medium and fortified with different concentrations of IBA (1, 2, 2.5, 5μM). The shoots were washed with water to remove agar attached to it, then dipped in bavistin for one min to avoid fungal contamination and thereafter transferred to rooting media**.** In case of liquid medium the microshoot was inserted in filter paper bridge which served as a support. The per cent response, number of roots and root length were recorded after four weeks.

All the experiments were repeated twice with five replicates. The data were analysed using ANOVA and mean separation were carried out using DMRT at 5% level of significance ($p = 0.05$).

Results and Discussion

In *Stereospermum suaveolens* studies were carried out for regeneration of shoots from nodal and cotyledonary node explants in WPM medium fortified with different individual cytokinins with various concentrations. Shoot cultures were successfully established from both the explants but the optimal media requirements varied.

WPM basal medium evoked a proliferation response in cotyledonary node in the form of single shoot but it failed to survive after third week. Thus the medium was fortified with individual cytokinin to overcome this problem. The presence of BAP/Kn/TDZ in the medium allowed the axillary bud to proliferate

and develop into shoot where morphogenic response was high in lower concentration as compared to high concentrations. It has been reported that percentage of shoot emergence decreases with increasing concentration of cytokinins (Reddy and Saritha 2013). In medium supplemented with BAP the concentrations (2 - 20 μM) stimulated axillary bud to proliferate and at 25 μM it failed to respond. The lower concentrations (4, 8 μM) evoked 100% response with only 1.2 ± 0.2 shoots per node which decreased to single shoot in 2, 16 and 20 μM (Table 1). Similar findings were observed in *Salix tetrasperma* (Khan et al. 2011) where BAP at lower concentration was effective in shoot proliferation.

When Kn was added in the medium, all the concentrations evoked a proliferation response but lower concentrations (2, 4 μM) induced an optimum percent response (100), In comparison to 4 μM, shoot emergence and its development was fast at 2 μM resulting in highest number of nodes 3.2 ± 0.3 (Table 1). At concentrations 8, 16 and 20 μM also single shoot formation was observed. Both BAP and Kn resulted into similar morphogenic response but shoots were longer in Kn as compared to BAP. Similar findings are reported in *Salvadora persica* (Kumari and Singh 2012) where Kn had a profound effect on length of shoots as compared to BAP.

TDZ was also tried, as it is known to be potent in inducing the axillary bud to proliferate and form multiple shoots in woody species (Huetteman and Preece 1993, Murthy et al. 1998). TDZ at all concentrations was able to stimulate the bud to proliferate and develop into a shoot, but lower concentration (0.1 μM) evoked an optimum response (83%) in terms of shoot formation. Observations revealed that in the presence of this TDZ (0.1- 2 μM) there was formation of profuse callus at the base of explant which hindered the growth of shoot. Similar results are reported in *Murraya koinegii* (Bhuyan et al. 1997) and *Oroxylum indicum* (Dalal and Rai 2004) where addition of TDZ resulted in heavy callus formation.

In cotyledonary node explant among all the cytokinins BAP and Kn were effective in terms of shoot induction as compared to TDZ. Cytokinins are known to play a key role in cell division and its presence in the medium is required for multiple shoot induction, but the type and its optimal concentration varies with the system (Park et al. 2008).

Nodal explants are, with vegetative buds, have a potential for rapid shoot regeneration and therefore in this species it was utilized to establish cultures. These explants were cultured in basal medium but resulted in poor proliferation response which improved when cytokinins were added to the medium. In BAP fortified medium (2 - 25 μM) an optimum per cent response (75) was observed at 16 μM (Table 2).

In Kn supplemented medium, all the concentrations were equally effective in shoot formation in which 2 µM proved to be optimum per cent response (80) and long healthy shoot with 2.8 ± 0.8 nodes were formed with well developed leaves. Observations revealed that presence of Kn in the medium induced an early response in terms of shoot formation when compared with other cytokinin. There are reports which state that Kn promotes elongation of buds in *Vigna radiata* (Chandra and Pal 1995).

Table 1. Effect of individual cytokinin on shoot bud initiation from cotyledonary node and nodal explants after four weeks in WPM Medium.

Cytokinins (µM)	Cotyledonary node explant			Nodal explant		
	% response	No. of shoots	No. of *in vitro* nodes	% response	No. of shoots	No. of *in vitro* nodes
0	50	0.5 ± 0.3^{abc}	0.5 ± 0.3^{a}	20	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}
BAP						
2	90	0.8 ± 0.1^{bc}	2.7 ± 0.7^{b}	80	1.2 ± 0.4^{a}	1.7 ± 0.5^{a}
4	100	1.2 ± 0.2^{c}	2.3 ±0.4^{b}	40	0.6 ± 0.4^{a}	1.1 ±0.7^{a}
8	100	1.2 ± 0.2^{c}	2.4 ± 0.4^{b}	80	1.4 ± 0.5^{a}	1.5 ± 0.6^{a}
16	71	0.4 ± 0.2^{ab}	1.3 ± 0.6^{ab}	75	1.0 ± 0.4^{a}	1.8± 0.6^{a}
20	50	0.6 ± 0.4^{abc}	0.5 ± 0.2^{a}	50	0.5 ± 0.3^{a}	1.5 ± 0.9^{a}
25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Kn						
2	100	1.0 ± 0.0^{bc}	3.2 ± 0.3^{c}	80	0.8 ± 0.2^{a}	2.8 ± 0.8^{b}
4	100	1.0 ± 0.0^{bc}	2.4 ± 0.4^{bc}	60	0.6 ± 0.2^{a}	1.8 ± 0.7^{b}
8	80	0.8 ± 0.2^{ab}	2.0 ± 0.5^{c}	50	0.5± 0.1^{a}	0.5 ± 0.2^{a}
16	50	0.5 ± 0.1^{a}	1.5 ± 0.5^{ab}	66	0.7 ± 0.3^{a}	1.7± 0.9^{ab}
20	83	0.8 ± 0.1^{ab}	2.7 ± 0.7^{bc}	75	0.8 ± 0.3^{a}	1.8 ± 0.8^{ab}
25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
TDZ						
0.1	83	0.7 ± 0.2^{a}	2.2 ± 0.7^{b}	80	1.0 ± 0.3^{b}	2.3 ± 0.7^{b}
0.2	50	0.5 ± 0.2^{a}	1.5 ± 0.7^{ab}	100	1.8 ± 0.8^{c}	3.6 ± 0.7^{c}
0.25	66	0.7 ± 0.2^{a}	2.3 ± 0.8^{b}	40	0.4 ± 0.2^{a}	1.8 ± 1.2^{b}
0.5	40	0.4 ± 0.2^{a}	0.6 ± 0.3^{ab}	10	0.1 ± 0.1^{a}	0.1± 0.1^{a}
1	40	0.4 ± 0.2^{a}	0.5 ± 0.2^{a}	10	0.1± 0.1^{a}	0.1± 0.1^{a}
2	50	0.5 ± 0.2^{a}	0.5 ± 0.2^{a}	20	0.2 ± 0.1^{a}	0.3± 0.2^{a}

Values represent mean ± SE of each experiment consist of five replicates conducted twice. Means values followed by different superscript letters within a column are significantly different at p = 0.05 according to DMRT.

The effect of TDZ was also evaluated and observations revealed that at 0.2 µM a 100% response was obtained which decreased with increasing concentration (0.25 - 2.0 µM). The reduction in the regeneration potential may be due to detrimental effect of high concentration on the cells predetermined to form vegetative buds (Khan et al. 2011). Presence of TDZ in the medium resulted in profuse callusing at the base of explants. The shoots which developed in 0.2 µM were with high number of nodes 3.6 ± 0.7 (Table 2) among all the three cytokinins

but remained stunted in growth. One of the reasons for suppressed growth of shoots may be due to high cytokinin activity of TDZ (Huetteman and Preece 1993). In the nodal explants the cytokinins induced similar morphogenic response, but the shoots developed were healthy as compared to cotyledonary node.

Table 2. Effect of IBA on root induction in WPM medium.

WPM		% response		No. of roots		Root length (cm)	
	IBA (μM)	Liquid	Static	Liquid	Static	Liquid	Static
Full strength	0	50	33	0.75 ± 0.5^{a}	1.0 ± 1.0^{a}	1.4 ± 0.8^{ab}	0.5 ± 0.5^{b}
	1	80	83	2.0 ± 0.5^{ab}	1.7 ± 0.3^{ab}	0.7 ± 0.3^{a}	0.3 ± 0.1^{ab}
	2	75	100	1.5 ± 0.9^{a}	3.7 ± 0.9^{b}	1.3 ± 0.4^{ab}	1.1 ± 0.3^{b}
	2.5	40	0	2.2 ± 1.4^{b}	0.0 ± 0.0^{a}	0.5 ± 0.4^{a}	0.0 ± 0.0^{a}
	5	40	40	1.6 ± 1.0^{ab}	3.8 ± 3.1^{b}	1.2 ± 0.8^{b}	0.1 ± 0.1^{a}
Half strength	0	50	66	0.75 ± 0.5^{a}	0.7 ± 0.3^{a}	4.9 ± 3.2^{b}	1.8 ± 1.4^{b}
	1	50	20	1.75 ± 0.5^{a}	0.2 ± 0.2^{a}	0.9 ± 0.8^{ab}	0.02 ± 0.02^{a}
	2	100	66	8.5 ± 3.1^{b}	0.7 ± 0.7^{a}	1.3 ± 0.8^{ab}	0.1 ± 0.1^{a}
	2.5	50	60	1.7 ± 0.8^{a}	1.2 ± 1.0^{a}	1.6 ± 0.8^{ab}	0.5 ± 0.4^{ab}
	5	75	75	2.0 ± 1.4^{a}	1.3 ± 1.3^{a}	0.2 ± 0.1^{a}	0.2 ± 0.2^{a}

Values represent mean ± SE of each experiment consist of five replicates conducted twice. Mean values followed by different superscript letters within a column are significantly different at α=0.05 according to DMRT.

Single *in vitro* nodes of both the explants were utilized as propagules for further multiplication of shoots by transferring them to fresh media with respective concentrations. Observations after eight weeks revealed that in both the explants there was slight increase in number of shoots in the medium fortified with only lower concentrations of BAP/Kn with increased shoot length whereas TDZ induced single shoots only.

The cotyledonary node explants failed to respond in cultures supplemented with high levels of BAP (16 and 20 μM) whereas at lower levels (2, 4 and 8 μM) only one to two shoots developed which considerably varied in their length from 4.7 ± 1.6, 3.5 ± 1.0 and 3.2 ± 1.5 cms, respectively. Presence of BAP in the medium could not enhance the number of shoots in further passages. Replacing BAP with Kn in the medium could induce morphogenic response at lower levels (2, 4 and 8 μM) whereas the axillary bud failed to respond at higher levels (16, 20 μM). At lower levels i.e. 2 μM the number of shoots reached 1.4 ± 0.2 with a length of 2.3 ± 0.3 cm (Fig. 1,a), whereas at 4 μM only single shoot with a length of 3.1 ±1.1 cm was formed and at 8 μM the number reached 1.5 ±0.4 with 2.0 ± 0.5 cm shoot length (Fig.1,b). TDZ at all concentrations failed in terms of shoot multiplication.

Therefore, Kn was effective for shoot multiplication in this explant and similar observations are reported for *Vigna* and *Gerbera jamesonii* (Sen and

Mukherjee 1998, Tyagi and Kothari 2004) but in *S. suaveolens* the shoots obtained were weak.

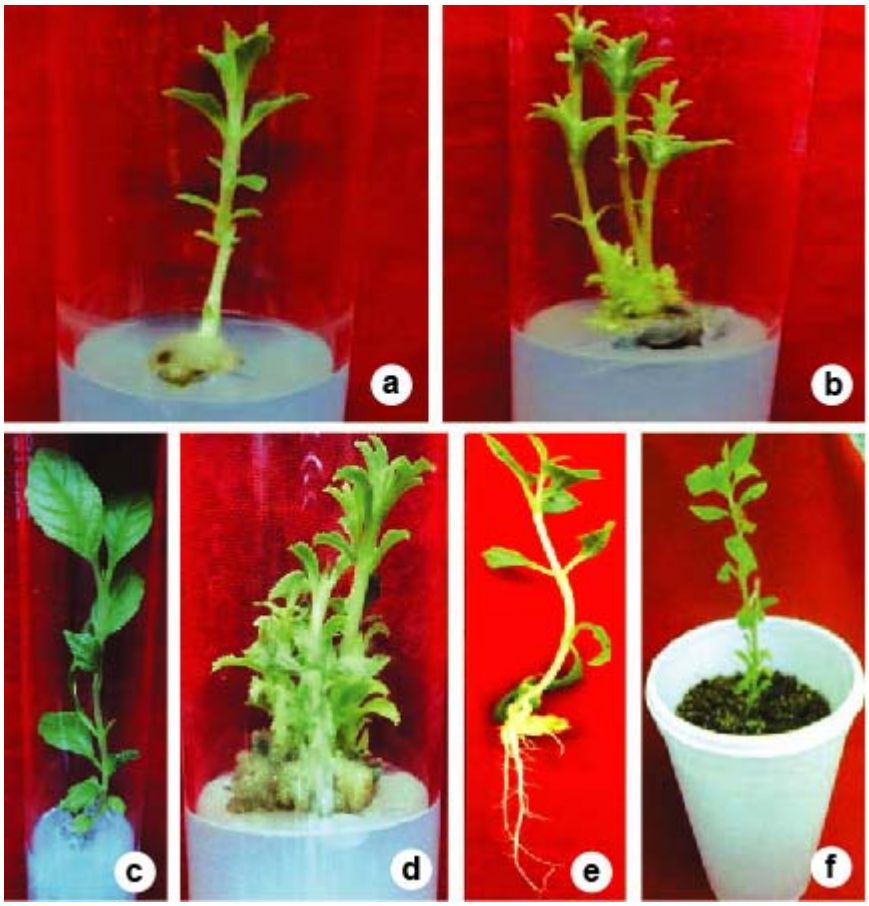

Fig. 1a-f: a. Shoot induction in WPM + Kn (2 μM) through cotyledonary node explant after eight weeks. b. Proliferation of multiple shoots in WPM + Kn (8 μM) through cotyledonary node explant after eight weeks. c. Single healthy shoot in WPM +Kn (2 μM) nodal explants after eight weeks. d. Multiple shoots induced from nodal explant in WPM + Kn (8 μM) after eight weeks. e. Root induction in liquid half WPM + IBA (2 μM). f. Plantlets acclimatized in cocopeat: sand : soil (1 : 1 : 1).

When the morphogenic response of nodal explants was evaluated, observations after eight weeks revealed that BAP and TDZ failed to respond in terms of shoot multiplication as in their presence only single shoot was produced. In Kn supplemented medium higher concentrations (16 and 20 μM) failed to form multiples shoot formation. This cytokinin with lower concentrations (2, 4 μM) developed one or two shoots only but the shoots were long (4.6 ± 0.4 and 4.7 ± 0.6 cms) and healthy with large leaves (Fig. 1c) which on further transferring to new media failed to form multiples. At 8 μM the number

reached 1.7 ± 0.5 and length was 2.0 ± 0.3 cm (Fig. 1,d). TDZ fortified medium developed into single shoots only with slight increase in length of shoots and at 0.1 μM it reached 3.3 ± 1.2 cm which on further transfer failed to respond.

In subsequent passages it was observed that Kn 8 μM was optimum in inducing multiple shoot with average number reaching 3.1± 0.5 and shoot length up to 3.8 ± 0.6 cm by the end of 12 weeks in cotyledonary node explants. But the shoots obtained were long and thin with very minute leaves. Compared to cotyledonary node the average shoot number was higher in nodal explants with 3.8 ± 0.6 shoots and length reaching 5.4 ± 0.4 after 12 weeks (Fig. 2). The shoots obtained from this explants were strong and healthy with well developed leaves as compared to cotyledonary node. Explant type has been known to effect multiple shoot induction in number of tree species like *Dalbergia sisoo* (Pradhan et al. 1998), *Pterocarpus marsupium* (Anis et al. 2005) and *Albizia odoratissima* (Rajeswari and Paliwal 2006).

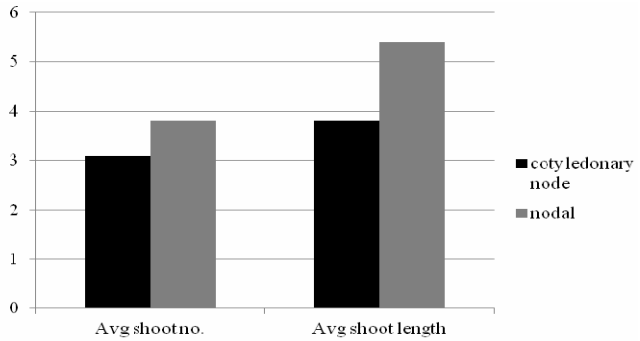

Fig. 2. Average shoot number and shoot length in cotyledonary node and nodal explants after 12 weeks in WPM + Kn (8 μM)

In the present studies the microshoots were transferred to full and half strength WPM (liquid and static) supplemented with IBA (0 - 5 μM). As it is a potent auxin for induction of roots (Mansor et al. 2003, Rajeswari and Paliwal 2008) and in woody trees usually low level of this growth regulator is effective for rooting of shoots (Rai et al. 2010). Root induction was observed in all the concentrations of both liquid and static media after four weeks, but half strength was superior to full strength WPM in terms of number of roots and their length. Similar results are reported in *Salix tetrasperma* also where half strength was superior to full strength WPM medium (Khan et al. 2011). After four weeks the root length was highest (4.9 ± 3.2 cm) in half strength basal liquid media but number of roots were less (Table 3) which were thin whitish in colour. Root induction in basal WPM has been reported earlier in *Salix* by Gebhardt (1992) and Park et al. (2008). The production of adventitious roots in medium without

auxin may be due to endogenous level of salicylic acid playing important role in plant growth development (Raskin 1992) and *in vitro* rooting (Khalafalla and Hattori 2000). Half strength liquid media with 2 μM concentration recorded optimum number of roots (8.5 ± 3.1) after four weeks (Fig. 1e). Same concentration was optimum in full strength static media with 3.7 ± 0.9 roots (Table 3). Zimmerman and Brome (1980) in blueberry noted that rooting can be obtained in solid and for better results in liquid media. In *S. suaveolens* also the liquid media obtained better results in terms of highest number of roots and root length compared to static media. Liquid media are known to be effective for rooting as the nutritive elements are easily available to explants (Hammerschlag 1982) and in static agar can create a critical pressure of turgescence which puts the cells in situation of stress.

The plantlets with well-developed roots were transferred to thermocol cups containing sterilized cocopeat, sand : soil (1 : 1), cocopeat: sand (1 : 1) and cocopeat : sand : soil (1 : 1 : 1) substrates and were covered with transparent perforated polybags. All the cups were kept in glass chamber placed in culture room at 25 ± 2°C. The plantlets were irrigated with MS basal medium and bavistin was sprayed on each substrate every alternate day to avoid fungal contamination. The mixture cocopeat : sand : soil (1 : 1 : 1) proved to be beneficial for the plantlets (Fig. 1f) as they started to grow and could survive even after four weeks while in others substrates the plantlets could not survive after second week. Further studies on hardening the plantlets in a suitable substrate are being carried out.

There are several factors like the type of medium, plant growth regulator and the explant which affects shoot regeneration as all are responsible in inducing an optimum morphogenic response. In our studies on *S. suaveolens* although both the explants were effective in establishing cultures the nodal explant was suitable in comparison to cotyledonary node for *in vitro* regeneration of shoots. Because nodal explants developed healthy plants compared to cotyledonary nodes. Out of the various combinations tried, WPM medium fortified with Kn 8 μM induced an optimum response for formation of multiple shoots and half strength liquid medium with IBA 2 μM was optimal for root induction in terms of number of roots. This protocol of regeneration through nodal explants is highly effective for clonal multiplication.

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