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BRIEF COMMUNICATION

Improved shoot regeneration system through leaf derived callus and nodule culture of *Sansevieria cylindrica*

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Abstract

Long-term culture establishment and efficient *in vitro* regeneration protocol for *Sansevieria cylindrica* Bojer *ex* Hook was developed using leaf derived callus and nodule culture. Profuse callus induction on leaf discs was achieved on Murashige and Skoog (MS) medium supplemented with 10 μM indole-3-butyric acid (IBA), while a high frequency of nodulation was induced on 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) containing media. Shoot regeneration ability from cultured tissues occurred at varying degrees on all media. Through callus culture a maximum of 17.6 ± 0.14 shoots per culture was formed on medium containing 5μM 6-benzyladenine (BA) and 2 μM α-naphthaleneacetic acid (NAA). Among nodule cultures, the 2,4-D generated nodules were more proliferative and regenerative as compared to 2,4,5-T induced nodules and a maximum of 25 ± 0.16 shoots per culture was produced on a medium containing 5 μM BA plus 1 μM NAA. The regenerated shoots were successfully rooted on a semi-solid half strength MS medium containing 5 μM IBA with an average root number 3.5 ± 0.18 and root length 6.5 ± 0.14 cm. The regenerative ability of callus tissues was steady upto one year, while the nodules retained the totipotency to regenerate on optimal medium even after 3 years of subculturing. The histological sections of nodules confirm the typical anatomy exhibiting the vascular elements in bundles with well demarcated cortex and epidermal covering.

Additional key words: auxins, cytokinins, multiple shoots, plant regeneration.

Among various agave species *Sansevieria cylindrica* is one of the most valuable and economic plant species described elsewhere (Anis and Shahzad 2005). Conventional propagation from leaf cuttings is very slow and time consuming and only a limited number of propagules can be obtained. Therefore, an efficient *in vitro* propagation technique certainly would provide an alternative means of producing large number of propagules of selected clone for further cultivation at an industrial level. There are several reports for an efficient regeneration through direct and indirect organogenesis in several agaves species; *Agave fourcroydes* (Robert *et al.* 1987), *A. cantala* and *A. sisalana* (Binh *et al.* 1990, Das, 1992, Nikam 1997), *A. parrasana* (Santacruz-Ruvalcaba *et al.* 1999), *Aloe barbadensis* (Sanchez *et al.* 1988), *Aloe polyphylla* (Abrie and Van Staden 2001) and recently a direct shoot regeneration protocol through leaf disc has been reported in *Sansevieria cylindrica* (Anis and Shahzad 2005).

Nodules are meristematic cell clumps containing independent, dense, spherical cells, which took part in a consistent internal cell and tissue differentiation (McCown *et al.* 1988). The aim of present experiments was establishment of callus cultures from leaf discs and regeneration of multiple shoots from nodules or callus mass. The long-term maintenance of the regenerative potential of these cultures was also determined.

Freshly emerging leaves of *Sanseveiria cylindrica*

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Abbreviations: BA - 6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA - gibberellic acid; IBA - indole-3-butyric acid; KIN - kinetin; MS medium - Murashige and Skoog medium; NAA - α-naphthaleneacetic acid; 2,4,5-T - 2,4,5-trichlorophenoxyacetic acid.

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(7 - 8 cm in length) were collected from the established stocks in the Botanical garden of the University. The explants were washed in running tap water for 30 min followed by 1 % *Bavistin* for 30 min, dipped in 5 % (v/v) *Teepol* (*Qualigens*, Mumbai, India) for 10 min, then rinsed with sterile double distilled water (DDW) and transferred to 70 % ethanol for 30 s. The source tissues were surface sterilized with 0.1 % (m/v) $HgCl_2$ for 7 min and thoroughly washed in sterile DDW. The basal portion (5 - 6 cm) of sterilized immature leaves were sliced to disc (5 mm thick) and used as explants for callus and nodule induction.

The culture media for callus induction and subsequent nodulation consisted of Murashige and Skoog (1962; MS) salts, 3 % sucrose and either indole-3-butyric acid (IBA; 1 - 15 μM), or 2,4-dichlorophenoxyacetic acid (2,4-D; 1 - 15 μM) or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; 0.1 - 1.5 μM). The pH of the medium was adjusted to 5.8 prior to addition of 0.8 % (m/v) agar (*Hi-Media*, Mumbai, India). All chemicals used were of analytical grade (*Sigma* or *Merck*). Medium was dispensed in 50-cm^3 culture tubes or 150-cm^3 flasks (*Borosil*, Mumbai, India). The media were autoclaved at 121 °C and 1.06 kg cm^{-2} pressure for 20 min. Nodules that developed on leaf disc were excised and transferred to fresh medium. The stock cultures thus obtained were routinely sub-cultured at monthly intervals for multiplication. The nodules developed on 2,4-D and 2,4,5-T and the calli developed on IBA were used as starting tissues for regeneration experiments.

For the induction of shoot organogenesis, the calli raised on IBA containing medium were sub-cultured on MS medium supplemented with 6-benzyladenine (BA; 1, 2 and 5 μM) in combination with α-naphthaleneacetic acid (NAA; 1 - 2 μM) or IBA (1 - 2 μM). The MS basal medium without growth regulators served as control. After harvesting the regenerated shoots, the stock callus was subcultured onto fresh regeneration medium after every 8 weeks. To test the long-term regenerative efficiency of callus tissue, sub-culturing was performed only onto optimal medium in every 4 weeks.

The nodules obtained on 2,4-D (10 μM) and 2,4,5-T (0.1 μM) containing media were separated and transferred onto regeneration medium comprised of BA or kinetin (KIN; 1 - 10 μM) along with NAA or IBA (1 μM). For long-term regeneration studies, the nodules were regularly sub-cultured on optimal medium.

The shoots measuring 2 - 3 cm were excised from cultures and planted onto rooting medium comprised of half strength MS medium with 3 % sucrose, augmented with IBA or NAA (1 - 5 μM). All the cultures were incubated in a growth chamber at 25 ± 1 °C , 16-h photoperiod with irradiance of of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (*Philips*, Kolkata, India) and 55 - 60 % relative humidity.

The plantlets having well developed root system were taken out from the culture vessels after 8 weeks of incubation, washed carefully with tap water, and transferred to pots containing *Soilrite*. The pots were covered with polythene bag to maintain high relative humidity for three weeks. After emergence of 2 - 3 new leaves, the plantlets were transferred to clay pots containing garden soil and kept under shade in a net house for another 2 weeks before transferring outside under full sun.

For histological study, the tissue with nodules were fixed in FAA (95 % ethanol + glacial acetic acid + formaldehyde + distilled water 10:1:2:7), dehydrated in tertiary butyl alcohol series, embedded in paraffin wax, sectioned at 10 μm thickness with rotary microtome (American Optical Corporation, Buffalo, USA) and stained with 0.5 % Safranin and 0.1 % Fast green (Johansen 1940).

Each treatment was replicated 10 times and all experiments were repeated thrice. The regeneration frequency, number of shoot buds per unit callus, conversion of shoot buds into shoot, shoot length, number of root and root length were recorded periodically. Data obtained were analyzed using one way analysis of variance (*ANOVA*) and means were compared using Tukey test at $P = 0.05$ with statistical software *SPSS* version *10* (*SPSS Inc*., Chicago, USA).

The leaf disc explants cultured on MS medium supplemented with IBA, 2,4-D (1, 5 and 10 μM each) and 2,4,5-T (0.1, 0.5 and 1 μM) started callusing within 15 - 20 d of incubation (Table 1). On IBA supplemented medium, callusing was more pronounced from the very beginning as compared to 2,4-D and 2,4,5-T where process of nodulation started after a slight callusing. IBA (10 μM) was found optimal for the induction of whitish loose callus which started after 10th day of inoculation. The calli were sub-cultured after 8 weeks onto the fresh medium of same composition for further proliferation before transferring onto regeneration medium. On higher IBA concentration, browning in callus was noticed within

Table 1. Effect of various auxins in different concentrations on percentage of responding explants, callus (- no, + poor, ++ moderate, +++ good) induction and nodule formation in *S. cylindrica* leaf disc culture on MS medium. Data taken after 8 weeks. Means followed by the same letter in each column are not significantly different ($P = 0.05$) according to Tukey test.

IBA [μM]	2,4-D [μM]	2,4,5-T [μM]	Response [%]	Callus	Number of nodules
1			0	-	0
5			20	++	0
10			80	+++	0
15			60	++	0
	1		30	+	1.4 ± 0.24^{d}
	5		60	+	9.0 ± 0.44^{b}
	10		90	++	17.0 ± 0.94^{a}
	15		80	+	9.6 ± 0.40^{b}
		0.1	50	+	2.2 ± 0.20^{d}
		0.5	80	++	10.6 ± 0.40^{b}
		1.0	70	++	5.8 ± 0.37^{c}
		1.5	60	+	3.2 ± 0.20^{d}

three weeks of its induction, while at lower concentration growth was considerably reduced. It appeared whitish, became hard in nature and did not organize to differentiate into shoots on regeneration medium.

Whereas, on both 2,4-D and 2,4,5-T containing media, nodules started to organize just after callus initiation after 15 d of incubation. Highest number of nodules (15 - 20) were observed on a MS medium with 10 µM 2,4-D, followed by that with 0.5 µM 2,4,5-T (Table 1, Fig. 1*A*). The nodules were dark green with rough withering epidermal cells. The transverse sections of compact greenish nodules demonstrated the presence of vascular elements in bundles with well demarcated cortex and epidermal covering (Fig. 1*E*,*F*) which was in consistence to the earlier report in poplar (McCown *et al.* 1988). The nodular tissue organization *in vitro* has been observed in many plant species, for instance eucalyptus (Warrag *et al.* 1991), pineapple (Teng 1997), lily (Godo *et al.* 1998), mangostem (Te-chato and Lim 2000), *Humulus lupulus* (Fortres and Pais 2000) and *Charybdis numidica* (Kongbangkerd *et al.* 2005), *etc*. The individual nodules were separated and cultured onto the same induction medium where they proliferated to a large number of small compact daughter nodules, which were used as explants for further regeneration studies.

The calli induced on IBA (10 µM) and the nodules obtained on 2,4-D (10 µM) and 2,4,5-T (0.5 µM) were cultured on MS medium amended with BA or KIN singly or in combination with NAA or IBA to evaluate the optimal medium for long-term establishment of cultures in a regenerative phase. Recently, similar study has been reported in *Capsicum baccatum* by Valera-Montero and Phillips (2005) who maintained the regenerative callus culture for at least 3 years on MS + BA + IAA + GA.

The leaf callus induced on MS medium containing IBA (10 µM) showed shoot bud regeneration when subcultured on a medium supplemented with BA in combination with NAA or IBA (Table 2). The MS medium devoid of growth regulator (control) did not support any morphogenic response. Organization of greenish smooth spots were observed from the surface of the callus after 10 d of sub-culturing, which were modified into shoot buds with the appearance of minute flat leafy outgrowth in the next 10 d. The highest regeneration frequency (100 %), maximum average number of shoots (17.6 ± 0.14) as well as shoot length (3.8 ± 0.14 cm) were achieved on a medium comprised of BA (5 µM) and NAA (2 µM) within 8 weeks of incubation. Similar to our results, in *Agave parrasana*, organogenic calli induced on MS medium supplied with 2,4-D differentiated into multiple shoots on MS medium fortified with BA and 2,4-D (Santacruz-Ruvalcaba *et al.* 1999). However, in *Agave sisalana* the embryogenic calli were produced from leaf explants on medium supplemented with 2,4-D, BA and GA_3 (Hazra *et al.* 2002). Nikam (1997) generated organogenic calli from stem and rhizome of *A. sisalana* on media comprised of MS + BA/KN + IAA/NAA. An attempts to induce differentiation in rhizome callus of *A. fourcryoides* with different auxins and cytokinins were also successful (Robert *et al*. 1987).

Upon transfer of nodules obtained on 2,4-D (10 µM) and 2,4,5-T (0.5 µM) to MS medium supplemented with various combinations of BA or KIN and NAA or IBA, regeneration of shoot buds was observed on all media and the numbers of regenerants ranged from 2.4 to 25.5 per culture (Table 3). The nodules without growth regulators did not support the regeneration. The shoot regeneration started after 3 weeks of incubation irrespective of the hormones used, but the response and the frequency of shoot regeneration was greatly influenced by the combination and concentrations of hormones. A best response of 2,4-D induced nodule was observed on BA

Fig. 1. *A* - Organization of nodules through leaf disc on MS + 1.0 µM 2,4-D. *B* - Multiplication and regeneration of nodules (induced on 2,4-D) cultured on MS + 5.0 µM BA + 1.0 µM NAA. *C* - Growth of regenerated shoot on MS + 5.0 µM BA + 1.0 µM NAA. *D* - Root induction in microshoot on ½ MS + 5.0 µM IBA. *E* - Histological section of nodule showing cortex and thick epidermal covering. *F* - Histological section of nodule showing the vascular bundle (*inset* showing the enlarged view of vascular bundle). *G* - Established plant in a pot.

Table 2. Effects of cytokinin and auxins on shoot regeneration from calli raised on IBA (10 μM). Data taken after 8 weeks. Means ± SE from 10 replicates. Means followed by the same letter in each column are not significantly different ($P = 0.05$).

BA [μM]	NAA [μM]	IBA [μM]	Response [%]	Number of shoots	Shoot length [cm]
0	0	0	0	0	0
1	1	-	60	2.6 ± 0.19^{g}	2.4 ± 0.17^{b}
2	1	-	80	6.7 ± 0.19^{e}	2.6 ± 0.17^{ab}
5	1	-	80	12.3 ± 0.16^{c}	2.9 ± 0.16^{ab}
1	2	-	80	8.6 ± 0.15^{d}	3.1 ± 0.16^{a}
2	2	-	100	14.6 ± 0.14^{b}	3.6 ± 0.14^{a}
5	2	-	100	17.6 ± 0.14^{a}	3.8 ± 0.14^{a}
1	-	1	30	1.4 ± 0.23^{h}	1.2 ± 0.14^{d}
2	-	1	40	2.3 ± 0.21^{gh}	1.8 ± 0.19^{c}
5	-	1	60	5.7 ± 0.19^{e}	3.2 ± 0.20^{a}
1	-	2	0	0	0
2	-	2	40	2.8 ± 0.18^{g}	1.8 ± 0.18^{c}
5	-	2	70	4.6 ± 0.17^{ef}	2.1 ± 0.19^{b}

Table 3. Effect of auxins and cytokinins on shoot regeneration percentage and number from nodules obtained on medium with 10 μM 2,4-D and 0.5 μM 2,4,5-T. Data taken after 8 weeks. Means ± SE from 10 replicates. Means followed by the same letter in each column are not significantly different ($P = 0.05$).

BA [μM]	KIN [μM]	NAA [μM]	IBA [μM]	10 μM 2,4-D [%]	shoot number	0.5 μM 2,4,5-T [%]	number
0	0		0	0	0		0
1	-	-	-	60	6.2 ± 0.15^{e}	40	5.1 ± 0.17^{c}
1	-	1	-	80	8.4 ± 0.16^{e}	55	5.4 ± 0.15^{c}
5	-	1	-	100	25.5 ± 0.16^{a}	75	15.3 ± 0.15^{a}
10	-	1	-	90	20.6 ± 0.14^{b}	70	12.4 ± 0.16^{b}
1	-	-	1	60	3.2 ± 0.19^{g}	55	2.5 ± 0.14^{ef}
5	-	-	1	65	12.4 ± 0.14^{d}	60	6.5 ± 0.17^{c}
10	-	-	1	60	10.5 ± 0.14^{de}	50	5.3 ± 0.17^{c}
-	1	-	-	30	2.4 ± 0.15^{g}	35	3.0 ± 0.22^{e}
-	1	1	-	45	6.4 ± 0.15^{e}	50	3.2 ± 0.23^{e}
-	5	1	-	65	13.6 ± 0.16^{cd}	55	3.6 ± 0.22^{e}
-	10	1	-	70	15.7 ± 0.16^{c}	60	4.1 ± 0.22^{d}
-	1	-	1	40	2.8 ± 0.15^{g}	35	2.4 ± 0.18^{f}
-	5	-	1	55	4.5 ± 0.14^{f}	50	2.6 ± 0.17^{ef}
-	10	-	1	60	7.8 ± 0.14^{e}	55	2.8 ± 0.17^{e}

(5 μM) + NAA (1 μM) with an average shoot number of 25.5 ± 0.16 (Table 3, Fig. 1*B*,*C*) as compared to 15.3 ± 0.15 from 2,4,5-T induced nodules on same medium. The increase in BA concentration (10 μM) did not affect much shoot regeneration efficiency, but at reduced concentration (1 μM) a considerable reduction in shoot number was evident (Table 3). The advantageous effect of BA over KIN as well as BA and NAA combination is well documented in various studies conducted in agaves, *A. cantala*, *A. fourcroydes*, *A. sisalana* (Binh *et al.* 1990; Nikam 1997), *Sansevieria cylindrica* (Anis and Shahzad 2005), and in other plants, *Yucca aloifolia* (Atta-Alla and Van Staden 1997), pineapple (Teng 1997,) and *Charybdis numidica* (Kongbangkerd *et al.* 2005).

To study the regenerability and stability of the stock tissue, subculturing has been done regularly at 4 weeks interval. The regenerating nodule tissues were sub-cultured on fresh medium where new nodules continued to appear and regenerated new shoot buds without losing their regeneration potentiality except that of slow growth in shoots. The earlier report of Lee *et al*. (2004) and Valera-Montero and Phillips (2005), are in agreement with our results and proves the regeneration ability of stabilizing long-term organogenic cultures, which retained the ability for more than 3 years.

Table 4. Effect of various auxins added to half-strenght MS medium on root induction in regenerated shoot of *S. cylindrica.* Data taken after 8 weeks. Means ± SE from 10 replicates. Means followed by the same letter in each column are not significantly different ($P = 0.05$).

IBA [μM]	NAA [μM]	Response [%]	Number of roots	Root length [cm]	Shoot length [cm]
0	0	40	1.6 ± 0.32^{c}	2.6 ± 0.15^{d}	4.2 ± 0.16^{b}
1	0	60	2.8 ± 0.19^{ab}	3.1 ± 0.15^{c}	4.7 ± 0.16^{b}
2	0	90	3.1 ± 0.19^{a}	4.8 ± 0.16^{b}	5.8 ± 0.14^{ab}
5	0	100	3.5 ± 0.18^{a}	6.5 ± 0.14^{a}	6.3 ± 0.15^{a}
0	1	50	2.2 ± 0.15^{abc}	3.4 ± 0.14^{c}	4.4 ± 0.18^{b}
0	2	75	2.5 ± 0.19^{ab}	4.2 ± 0.16^{b}	4.4 ± 0.17^{b}
0	5	80	2.8 ± 0.17^{ab}	4.2 ± 0.16^{b}	4.6 ± 0.19^{b}

Root initiation took place within 8 - 15 d and optimum rooting (100 %) with maximum number of roots (3.5 ± 0.18) and root length (6.5 ± 0.14 cm) was obtained on IBA (5 μM) incorporated into half strength MS medium (Table 4, Fig. 1*D*). Fast growing thick roots were produced on IBA containing medium, while the NAA supplemented medium induced thin delicate roots with slow growth and resulted in some callus formation which is considered to be undesirable for *ex vitro* establishment. This type of undesirable response was also observed in previous study in *S. cylindrica* (Anis and Shahzad 2005). The treatment of IBA was most effective in some agaves like *Aloe parrasana* (Santacruz-Ruvalcaba *et al.* 1999), *A. polyphylla* (Abrie and Van Staden 2001, however, for many other agave studies only basal medium was sufficient for root induction (Binh *et al.* 1990, Das, 1992). In some other plants, *e.g.*, *Argyrolobium roseum* (Khanna *et al.* 2006), *Tylophora indica* (Faisal and Anis 2005) also reported the higher frequency of root induction on medium with IBA than with NAA or IAA. The reduction in nutrient supply for better effect in rooting efficiency as described in the present study is in consonance with the previous reports made by Rao and Purohit (2006) in *Celastrus paniculatus* and Sujatha and Kumar (2007) in *Carthamus tinctorious*. The rooted

shoots were taken out from the culture vials (after 8 weeks) and acclimatized successfully in *ex vitro* conditions with 90 % survival rate as described earlier and the established plant exhibits the normal developmental pattern and flowering (Fig. 1*G*).

The results reveal that micropropagation of *S. cylindrica* through callus as well as nodule culture is feasible for mass production of plantlets for field cultivation. Due to the potential of automation of callus and nodule-based propagation system, plant production through shoot organogenesis is an attractive alternative to other regeneration pathways

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