Journal of Phytology 2012, 4(3): 40-44
ISSN: 2075-6240
Available Online: http://journal-phytology.com/

Rapid callogenesis and plant regeneration from nodal explants of *Sida cordifolia* L.- an important medicinal plant

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Abstract
An efficient reproducible protocol for rapid callus induction and plant regeneration from young nodal explants of *Sida cordifolia* L. was developed. High frequency of green, compact organogenic callus was obtained from nodal explants cultured on MS medium supplemented with 0.5 mg/L Kinetin individually and combination with 0.5 mg/L NAA and successful plant regeneration from *in vitro* derived callus of *Sida cordifolia* L. Kn and BAP individually and in combinations with NAA and IAA used for regeneration of plantlets from callus culture. Maximum number of multiple shoots were developed on MS medium supplemented with 0.5 mg/L KN+0.5 mg/L NAA. All the *in vitro* shoots were then transferred to rooting medium supplemented with different concentrations of 0.2-1.0 mg/L NAA and IAA individually. The best rooting response was observed on 0.8 mg/L NAA. The well rooted plantlets were transferred to polybags containing soil + sand + vermiculites (2:1:1) for hardening. Finally the hardened plantlets were transferred to fields for acclimatization.

Keywords: *Callus induction, Kinetin, NAA, Nodal explant, Plant regeneration, Sida cordifolia* L.

INTRODUCTION

Sida cordifolia L. (Malvaceae) is commonly known as Bala (Sanskrit) and Country Mallow (English). The whole plant is used as traditional medicine, because, it contains alkaloid-ephedrine, vasicinol, vasicinone and N-methyl tryptophan [1,8,9,15]. This plant used as traditional medicine against chronic dysentery, asthma, and gonorrhea in the Indian subcontinent [3,26]. Recently, cardiovascular effects [18], analgesic, antiinflammatory [25] and hypoglycemic activities [13] were reported from its leaves. Because of these important medicinal properties, *S. cordifolia* L. is under threat due to extensive and unscientific collection and continuous deforestation. This requires micropropagation and *in vitro* conservation of this traditional medicinal plant for the future generation, pharmacological studies and genetic improvement programs. Considering its high medicinal values and pharmacological importance of secondary metabolites, industries are deeply interested in utilizing plant tissue culture technology for large scale production of these substances [19]. Hence, the present investigation was undertaken to develop protocol for *in vitro* multiplication of *S.cordifolia* L.

MATERIALS AND METHODS

Collection of Plant material

Sida cordifolia L., plants were collected from the Karnatak University campus Dharwad. Collected plant materials grown and maintained in the garden of Department of Botany, Karnatak University, Dharwad, Karnataka, India. for source of explants.

Received: Jan 12, 2012; Revised: Feb 20, 2012; Accepted: March 17, 2012.

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Surface sterilization

Nodal segments (0.5-1.0 cm) of young healthy plants were used for *in vitro* culture. The nodal explants were first thoroughly washed under running tap water for 15-20 minutes and then treated with liquid detergent (Tween-20) for 5-10 minutes. Later, these explants were washed with double distilled water for 5 minutes. Subsequently, explants immersed in 70% (v/v) ethanol for 2-3 minutes and washed with sterile glass double distilled water for 2-3 times. Eventually, the explants were treated with aqueous solution of 0.1% (w/v) $HgCl_2$ for 1-2 minutes and rinsed with sterile glass double distilled water for 2-3 times to remove traces of mercuric chloride. The sterilized explants were aseptically inoculated on to MS Medium with different combination and concentrations of hormones.

Culture media and growth condition

MS medium with 3 % (w/v) sucrose and pH 5.6-5.8 is solidified with 0.8 % agar prior to autoclave at 121^0C at 15 lbs pressure for 15-20 minutes is used. Inoculations were done under aseptic conditions in a laminar air flow cabinet, where cultures tubes (150 X 25 mm) containing 20-25 ml medium and plugged tightly with non-absorbent cotton kept under UV light for sterilization before inoculation for 30 minutes. All culture tubes were incubated in a controlled-environmental growth chamber at $25^0 \pm 2^0$ C under 16-18 hrs photoperiod at 3,000 lux light intensity (40W white fluorescent tubes, Philips, India.) and with 55-60 % relative humidity.

Sub culturing

The cultures were maintained by regular subculture at 4-6 weeks intervals on fresh MS medium.

Data collection and statistical analysis

The data for percentage of response per explants, regarding number of shoots, shoot length, number of roots and root length was recorded after six weeks of culture. The data were analyzed statistically using SPSS.16. The significance of difference among the means was calculated using Duncan's Multiple Range Test.

RESULTS

Young nodal explants of *Sida cordifolia* L. were cultured on MS medium supplemented with different concentrations of cytokinins (Kn and BAP) individually and in combination with auxins *(*NAA and IAA). Green, compact callus was developed from nodal explants after 4 weeks of incubation.

Effect of Kn and BAP individual or in combinations with IAA on callus induction

Callus induction was observed on MS medium supplemented with different concentrations of Kn and BAP individually and in combinations with IAA. Initially light green callus was developed from the cut portions of nodal explants after 15-20 days of incubation. Depending on the concentrations and combinations of various hormones used frequently for callus formation and different types of calli were observed. At lower concentration of 0.2 mg/L Kn light brown callus, and in higher concentration (1mg/L) dark brown callus was formed. Colour complexity of callus turns from brown to light green after subculture on MS medium with 0.5 mg/L Kn and 0.5 mg/L IAA. (Table.1).

Effect of NAA and IAA in combinations with Kn and BAP on induction of callus

Callus initiation was also observed on MS Medium supplemented with different auxins (NAA, IAA) individually and in combinations with cytokinins (Kn and BAP). In the combinations of Kn and NAA on MS medium callus was induced after 2 weeks of incubation from young nodal explants. Subsequently, the mass of callus was increased rapidly after another 2 weeks of incubation in the same medium. In the combinations of Kn and NAA, profuse green, compact, organogenic callus was developed from nodal explants (Table.1; Fig.1A). Among the different combinations of cytokinins and auxins used, 0.5 mg/L Kn and 0.5 mg/L NAA, proved as better for rapid induction of green, compact, organogenic callus. (Fig.1.B).

Table 1. Effect of different concentrations of cytokinins (Kn and BAP) individually and in combinations with auxins (NAA and IAA) on induction of callus from young nodal explants of *Sida cordifolia* L.

Plant Growth Regulators (mg/L)				Intensity of callus induction	Nature of callus
Cytokinins		Auxins			
Kn	BAP	NAA	IAA		
0.1				-	No callus induced
0.2				+	Light brown, fragile
0.5				++	Light green, compact
0.8				+	Light green, fragile
1.0				+	Yellowish green, nodular
1.5				+	Yellowish green fragile
0.5	0.1			+	Light brown, fragile
0.5	0.2			+	Yellowish green, fragile
0.5	0.5			++	Light green, nodular
0.5	0.8			+	Light green, fragile
0.5	1.0			+	Light brown, fragile
0.5	1.5			+	Light brown, fragile
0.5		0.1		+	Light green, nodular
0.5		0.2		++	Light green, compact, organogenic
0.5		0.5		+++	Dark green, compact, organogenic
0.5		0.8		++	Dark green, compact, nodular
0.5		1.0		+	Light green, nodular
0.5		1.5		+	Yellowish green, nodular
0.5			0.1	+	Yellowish green, fragile
0.5			0.2	+	Creamish brown, fragile
0.5			0.5	++	Yellowish green, creamy, fragile
0.5			0.8	+	Light brown, fragile
0.5			1.0	+	Whitish green, fragile
0.5			1.5	+	Light brown, fragile

Note: Intensity of callus: += low; ++= moderate; +++= high.

In vitro plant regeneration from nodal callus
Effect of Kn and BAP for multiple shoots regeneration

Well profused green, compact callus derived from young nodal explants was sub-cultured on fresh MS medium supplemented with different concentrations of Kn and BAP individually. After four weeks of subculture, subsequently multiple shoots were induced from nodal callus. MS medium supplemented with Kn at lower concentration (0.1mg/L), the frequency of shoot initiation was lower (45%), further, increase in Kn concentrations (0.1 mg/L<) enhanced the frequency of shoot induction. High frequency (90 %) and maximum number of multiple shoots were induced on MS medium supplemented with 0.5 mg/L Kn (Table.2). Furthermore, MS medium supplemented with 0.5 mg/L BAP individually, the explants showed reduced frequency (70 %) (Table.2).

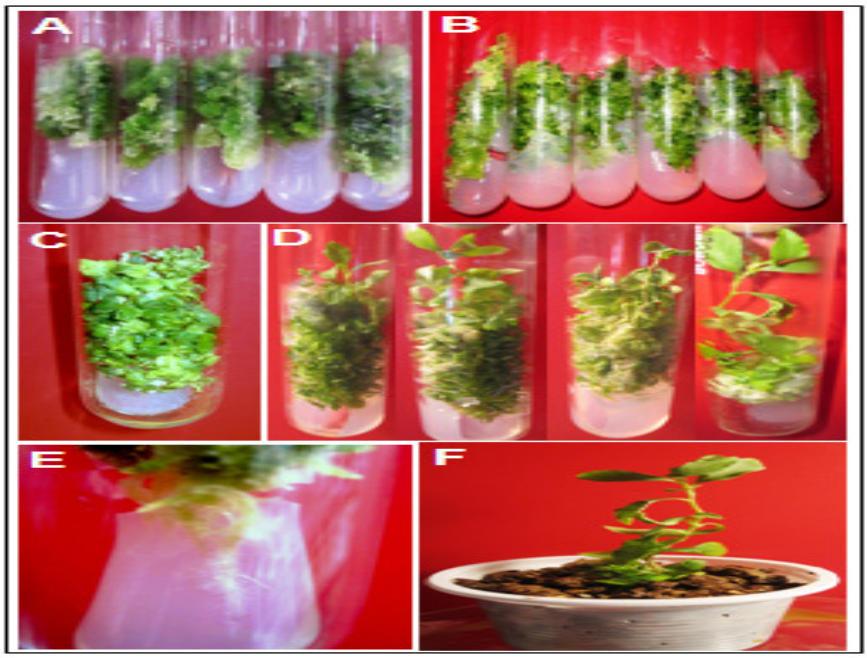
Effect of Kn and auxins (NAA and IAA) combinations on indirect shoot regeneration

The presence of cytokinins along with auxins is necessary for indirect multiple shoot induction. Maximum Number of multiple shoots induced on MS medium supplemented with 0.5 mg/L Kn was 95 % and highest shoot length was observed on MS medium supplemented with 0.5 mg/L Kn and 0.5 mg/L NAA (Table.2; Fig.1C). When MS medium supplemented with 0.5 mg/L Kn and 0.5 mg/L IAA showed 70 % of multiple shoots (Table.2). In the present study, MS medium supplemented with cytokinin (Kn) in combination with Auxin (NAA) has induced maximum number of multiple shoots.

Table 2. Effect of different concentrations of cytokinins (Kn and BAP) individually and in combinations with auxins (NAA and IAA) on multiple shoot regeneration from nodal callus of *Sida cordifolia* L.

Growth regulators (mg/L)				Frequency of shoot induction (%)	Number of shoots / culture (Mean±SE)	Shoot length/ culture (cm) (Mean±SE)
Kn	BAP	NAA	IAA			
0.1				45	1.8 ± 0.56^{d}	0.48 ± 0.12^{b}
0.2				65	4.5 ± 1.06^{c}	0.50 ± 0.15^{b}
0.5				90	18.2 ± 0.15^{a}	1.70 ± 0.12^{a}
0.8				80	14.0 ± 0.74^{b}	0.60 ± 0.14^{a}
1.0				75	8.9 ± 0.83^{c}	0.38 ± 0.05^{c}
	0.1			30	0.8 ± 0.58^{b}	0.12 ± 0.10^{e}
	0.2			45	2.0 ± 0.40^{b}	0.14 ± 0.1^{d}
	0.5			70	3.9 ± 0.86^{a}	0.80 ± 0.1^{a}
	0.8			55	3.8 ± 0.94^{a}	0.82 ± 0.1^{b}
	1.0			40	3.5 ± 1.16^{a}	0.71 ± 0.1^{c}
0.5		0.1		55	2.5 ± 0.67^{c}	1.1 ± 0.06^{e}
0.5		0.2		80	6.8 ± 1.01^{b}	1.2 ± 0.01^{d}
0.5		0.5		95	16.2 ± 0.35^{a}	3.0 ± 0.02^{a}
0.5		0.8		80	8.5 ± 0.50^{b}	1.8 ± 0.03^{b}
0.5		1.0		75	6.2 ± 0.54^{b}	1.5 ± 0.02^{c}
0.5			0.1	35	2.1 ± 0.55^{c}	0.56 ± 0.02^{c}
0.5			0.2	50	2.0 ± 0.37^{b}	0.60 ± 0.01^{b}
0.5			0.5	70	4.0 ± 0.24^{a}	0.70 ± 0.02^{a}
0.5			0.8	55	4.5 ± 0.37^{b}	0.58 ± 0.01^{b}
0.5			1.0	45	3.2± 0.31^{c}	0.55 ± 0.01^{c}

Each value represents the means ± SE of 10 replications.
Values followed by the same letter within columns are not significantly different at P≤0.05.



A. Induction of callus from nodal explants in MS+0.5 mg/L Kn +0.5 mg/L NAA after 4 weeks of incubation.
B. Proliferation of multiple shoots after 6 weeks of incubation.
C. Multiple shoot developed on MS+0.5 mg/L Kn + 0.5mg/L NAA after 8 weeks of incubation.
D. Multiple shoots formation and elongation after 12 weeks of incubation.
E. *In vitro* roots on MS+0.5 mg/L Kn + 0.8 mg/L. NAA.
F. Hardening and Acclimatization of *in vitro* plantlet.

Fig 1. Rapid callogenesis and plant regeneration from nodal explants of *Sida cordifolia* L.

In vitro rooting

In vitro shoots were excised (2-5 cm) and inoculated on MS medium supplemented with different concentrations of 0.1-1.5 mg/L NAA (Fig.2A). MS medium supplemented with NAA, the number of roots, root length were high compare to MS medium supplemented with IAA. Maximum number of roots (62.7±0.05) were induced on MS medium supplemented with 0.8mg/L NAA (Table.3).

Table 3. Effect of NAA and IAA on root induction from *in vitro* shoots of *Sida cordifolia* L.

Auxins concentrations (mg/L)		Frequency of root induction (%)	Number of roots/shoot (Mean ± SE)	Root length / culture (cm) (Mean ± SE)
NAA	IAA			
0.1		35	2.4±0.17^{e}	0.3±0.01^{e}
0.2		40	8.4±0.11^{d}	0.5±0.01^{d}
0.5		60	18.6±0.14^{b}	1.2±0.02^{b}
0.8		90	62.7±0.05^{a}	3.5±0.03^{a}
1.0		45	12.4±0.17^{c}	0.8±0.03^{c}
	0.1	30	1.9±0.05^{e}	0.1±0.01^{d}
	0.2	35	5.2±0.12^{d}	0.2±0.01^{c}
	0.5	45	9.6±0.21^{b}	0.4±0.01^{b}
	0.8	50	15.0±0.26^{a}	0.7±0.02^{a}
	1.0	25	8.0±0.57^{c}	0.2±0.01^{c}

Each value represents the means ± SE of 10 replications.
Values followed by the same letter within columns are not significantly different at P≤0.05.

Hardening and Acclimatization

Well rooted plantlets were separated, washed and transferred from the culture tubes to polybags containing soil + sand + vermiculites (2:1:1) for hardening. Finally, the hardened plantlets were transferred to field conditions for acclimatization (Fig. 2 B).

DISCUSSION

After 4 weeks of culture, rapid callus was induced from nodal explants when cultured on MS medium supplemented with different concentrations of different plant hormones. Callus was initiated after 8-10 days of incubation from the cut end of the nodal explant cultured in the MS medium supplemented with Kn, BAP, NAA and IAA individually and in combinations (0.1 to 1.0 mg/L). Callus initiation was from the cut end portions of the nodal explant may be due to wound reaction or effect of exogenous growth regulators. The texture of callus varied according to the nature of cytokinins and also on auxin:cytokinin ratio in *Holostemma ada-kodien* [17].

In the present study, high rate of callus growth was induced on MS medium supplemented with Kn individually and combinations with NAA. The combination of cytokinins and auxins was reported to stimulate *in vitro* multiplication and growth of shoots in several plant species. [7]. Light green callus was induced in 0.5 mg/L Kn only, then combination with 0.5 mg/L NAA potentially dark green, compact, organogenic callus was induced. Similar results were reported in *Picrorhiza kurroa* on MS medium with 4.0 mg/L NAA and 1.0 mg/L Kn. [16].

Callus developed on MS medium supplemented with 0.5 mg/L Kn + 0.5 mg/L BAP was yellowish green, nodular and showed the limited growth. Though the callus was induced even on MS medium supplemented with 0.5 mg/L IAA + 0.5 mg/L Kn induced the quantity was less and it was yellowish green, creamy, fragile and failed to multiply further. However callus obtained on MS medium supplemented with 0.5 mg/L Kn and 0.5 mg/L NAA was turned creamy translucent and crystallized mass was turned to light green and modified into hard, compact green solid mass of callus which was multiplied even up to 50 to 70 folds in 40 days. Callus thus grown had high regenerative potential. This was proved when, multiple shoots were obtained on the same medium by subjecting same callus for incubation for two more weeks. Cytokinin along with auxin is necessary for indirect shoot induction as noted by Skoog and Miller (1957) it is also true with present study on *S. cordifolia* L.

Shoot differentiation was observed from the callus after 4 weeks of subculture on MS medium with 0.5 mg/L Kn + 0.5 mg/L NAA (Figure 1C). Callus proliferation with high frequency multiple shoots were regenerated on the same MS medium after 12 weeks (Figure 1D). Sivanesen and Byoung (2007) achieved direct shoot regeneration from nodal explants of *S. cordifolia* L. without intervening of callus on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA. Lal and Ahuja (1996) maintained and proliferated calli of *Picrorhiza kurroa* on MS medium containing 4.0 mg/L NAA and 1.0 mg/L Kn for 2 weeks. Martin (2002) observed callus from nodal explants of *Holostemma ada-kodien* and he suggested that the induction of callus and the regeneration of multiple shoots from nodal explants may be due to the presence of some internal components from the pre-existing axillary buds that are essential for induction of caulogenesis. Karnawat *et al*. (2011) supported the view of Martin (2002) after inducing high multiple shoots from nodal explants of *Verbesina encelioides* on MS medium supplemented with 3 mg/L BAP. They claimed that the *in vitro* regeneration was more successful from axillary buds than by indirect organogenesis. Regenerated multiple shoots were elongated on the same medium in *S.cordifolia* L. the present work, and continued for two sub-cultures at an interval of 15 days. Similar studies on *Leptadenia reticulata* [5] and *Hypericum maculatum* [2] reported indirect organogenesis from nodal explants.

Use of auxins individually or in combinations for induction of roots was reported for medicinal plants *Eclipta alba* [10] *Picrorhiza kurroa* [11] and *Ceropegia spiralis* [24]. In the present study also roots were induced using higher concentration of auxin in combination with cytokinin 0.8 mg/L NAA +0.5 mg/L Kn. Instead of NAA, IAA was effective in induction of roots from *in vitro* grown shoots of *Ilex khasiana* [4]

CONCLUSION

Some of medicinal plants are being depleted rapidly due to overexploitation and unscientific method of collection. Hence, an efficient reproducible protocol was developed for rapid callus induction and *in vitro* plant regeneration from nodal explants of *Sida cordifolia* L. This will be used to make the plant available throughout the year for the traditional healers, pharmaceutical usages, the establishment of germplasm conservation, commercial cultivation and also for secondary metabolite production from callus sources.

ACKNOWLEDGEMENT

The authors are grateful to the UGC (New Delhi, India) for financial assistance (RFSMS) and Chairman P.G Dept. of Botany for providing necessary facilities.

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