Rapid *in vitro* regeneration of *Sesbania drummondii*

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Abstract

This paper describes rapid propagation of *Sesbania drummondii* using nodal explants isolated from seedlings and young plants. The nodal segments proliferated into multiple shoots on Murashige and Skoog's (MS) medium supplemented with 22.2 μM benzyladenine. MS medium containing 2.2 and 4.5 μM thidiazuron induced 5 - 6 shoots per stem node from 3-month-old plants. Nodal explants when cultured on MS medium containing combinations of benzyladenine (8.8 and 11.1 μM) and indole-3-butyric acid (0.24 - 2.46 μM) or indole-3-acetic acid (0.28 - 2.85 μM) gave lesser number of shoots. Callus induced on cotyledonary explants when subcultured on 2.2 μM thidiazuron containing medium resulted in its mass proliferation having numerous embryoid-like structures. Indole-3-butyric acid (0.24 - 2.46 μM) was found suitable for root induction. *In vitro* regenerated plants were acclimatized in greenhouse conditions.

Additional key words: 6-benzyladenine, indole-3-acetic acid, indole-3-butyric acid, medicinal plant, metal hyperaccumulator, micropropagation, thidiazuron.

Introduction

Sesbania drummondii (Rydb.) Cory (*Fabaceae*) is a perennial shrub. It is distributed in seasonally wet places of southern coastal plains of the United States of America, from Florida to Texas. Seed extracts of this plant are significantly active against lymphocytic leukemia P-388 *in vivo.* This was attributed to the presence of medicinally important alkaloids: sesbanimide and sesbanine (Powell and Smith 1981). Besides being a source for the various pharmaceutically valuable compounds, *Sesbania drummondii* is a hyperaccumulator of toxic heavy metals like lead, copper and zinc (Sahi *et al*. 2002).

Of a wide range of *Sesbania* species, *in vitro* regeneration protocols have been developed for *S. rostrata* (Vlachova *et al*. 1987), *S. sesban* (Khattar and Mohan Ram 1982, Harris and Puddephat 1989, Zhao *et al.* 1993), *S. grandiflora* (Khattar and Mohan Ram 1983, Shanker and Mohan Ram 1990), *S. cannabina* (Xu *et al*. 1984, Shahana and Gupta 2002), *S. bispinosa* (Kapoor and Gupta 1986, Sinha and Mallick 1991). In these species, plant regeneration has been obtained by enhanced axillary branching as well as callus-mediated organogenesis from seedling explants and greenhouse grown plant-parts. There is no systematic cultivation of this plant and no published reports on tissue cultures of *Sesbania drummondii* are available. Therefore, there is a need to develop a means for rapid regeneration of plantlets. The present study thus focuses on *in vitro* morphogenesis and plant regeneration using various seedling explants such as cotyledonary node, axillary branch node, hypocotyl, epicotyl, cotyledon, and stem segments from greenhouse-grown plants.

Materials and methods

Seeds of *Sesbania drummondii* were scarified using 85 % H_2SO_4 for 30 min followed by washing under running tap water for 1 h. Seeds were sterilized with 0.2 % $HgCl_2$ and then transferred to magenta boxes containing water-agar

Received 3 March 2003, *accepted* 15 January 2004.

Abbreviations: BA - 6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron; MS medium - Murashige and Skoog's (1962) medium.

Acknowledgements: Authors wish to acknowledge the Biotechnology Center, the Department of Biology and Western Kentucky University for their financial support.

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(0.6 %), and incubated at 25 - 28 °C under 16-h photoperiod of 20 μmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes.

Ten-day-old seedlings were harvested for use of different explants: CN (cotyledonary node: 1.5 - 2 cm segment of embryonic axis bearing nodes without cotyledons), AN (axillary branch node), hypocotyl and epicotyl. Individual cotyledons were excised from sterilized seeds soaked for 48 h on water-agar medium. Stem segments (bearing nodes) excised from 3-month-old greenhouse-grown plants were also used. Explants were surface sterilized with 0.1 % $HgCl_2$ for 5 min and then rinsed three times with sterile deionized water.

Murashige and Skoog (1962; MS) medium was supplemented with various concentrations of plant growth regulators: 4.4 - 22.2 μM benzyladenine (BA); 8.8 or 11.1 μM BA + 0.24 - 2.46 μM indole-3-butyric acid (IBA); 8.8 or 11.1 μM BA + 0.28 - 2.85 μM indole-3-acetic acid (IAA); 4.4 - 22.2 μM BA + 1.34 - 8.0 μM naphthaleneacetic acid (NAA) and 2.27 - 4.54 μM thidiazuron (TDZ). The basal (MS) medium also contained 30 g dm^{-3} of sucrose and 8 g dm^{-3} agar as gelling agent; the pH of the media was adjusted to 5.8 after adding plant growth regulators. After sterilization (121 °C, 20 min) medium (15 cm^3 each) was dispensed into culture tubes (150 × 25 mm), which were plugged with caps (*Fisher Scientific Co*., Pittsburg, USA). Explants were transferred to culture tubes containing different concentrations of plant growth regulators. These cultures were incubated at 25 ± 2 °C under 16-h photoperiod of 50 μmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes for 35 d. Each experiment consisted of 12 explants (in different culture tubes) and was repeated twice. The cotyledons excised from imbibed (48 h) seeds were cultured on MS medium containing different concentration of BA (2.2 - 13.3 μM). Calli were sub-cultured on MS medium containing 2.27 μM TDZ at every 4 - 5 weeks.

Shoots regenerated from stem nodes (from 3-month-old plants) on TDZ medium were excised and elongated individually on MS medium supplemented with 1.14 μM IAA. Nodal segments from elongated shoots were transferred to MS medium containing 2.27 μM TDZ for stage II multiplication.

For rooting, 4 - 5 cm long regenerated shoots were transferred to MS medium alone and in presence of 0.24 - 2.46 μM IBA as well. After plantlets attained height of 8 - 10 cm, they were transferred to plastic cups containing autoclaved peat moss and covered with plastic wrapper to prevent humidity loss. Plants were maintained at 25 ± 2 °C and 60 - 70 % relative humidity under bright daylight. Plants were frequently watered and gradually exposed to the natural environment.

Statistical analysis of data was carried out by using *SYSTAT* (*version 9.0 for Windows*, *Systat Software Inc*. Richmond, USA). Observations were recorded for the frequency (percent of cultures responding to shoot proliferation, callusing and root development) and the number of shoots per explant, and shoot length. The analysis of variance (*ANOVA*) appropriate for the design was carried out to detect the significance of differences ($P < 0.05$) among the treatment means and Tukey HSD *post hoc* test was performed to compare among the groups for significant differences.

Results and discussion

Axillary branch node (AN) and cotyledonary node (CN) segments of 10-d-old seedlings proliferated into multiple shoots on MS medium supplemented with 4.4 - 22.2 μM BA. With increase in BA concentration, number of shoots

Table 1. Shoot proliferation from S. *drummondii* cotyledonary node (CN) and axillary branch node (AN) explants (excised from seedlings) and stem node (excised from 3-month-old plants; SN) following 5 weeks of culture on different concentrations of BA and TDZ. Each mean is based on three sets of replicates, each of which consisted of 12 culture tubes. Means having different letters in superscript are significantly different from each other ($P < 0.05$) according to *ANOVA* and Tukey HSD multiple comparisons *post hoc* test.

BA [μM]	TDZ [μM]	Shoot length [cm]			Number of shoots [explant^{-1}]			Regeneration [%]		
		CN	AN	SN	CN	AN	SN	CN	AN	SN
4.40		2.5^{a}	1.5^{a}		2.63^{a}	2.18^{a}		90	90	
8.80		2.5^{a}	1.0^{b}		5.00^{b}	3.58^{b}		90	90	
11.10		2.0^{ca}	1.0^{b}		4.75^{b}	3.83^{b}		100	100	
13.30		2.0^{ca}	0.7^{c}		6.50^{c}	4.08^{c}		90	90	
18.50		1.5^{b}	0.5^{d}		7.08^{d}	2.66^{b}		80	80	
22.50		1.5^{b}	0.5^{d}		7.75^{d}	1.50^{a}		80	80	
	2.27			2.7^{a}			6.20^{a}			60
	4.54			2.5^{a}			5.00^{b}			50

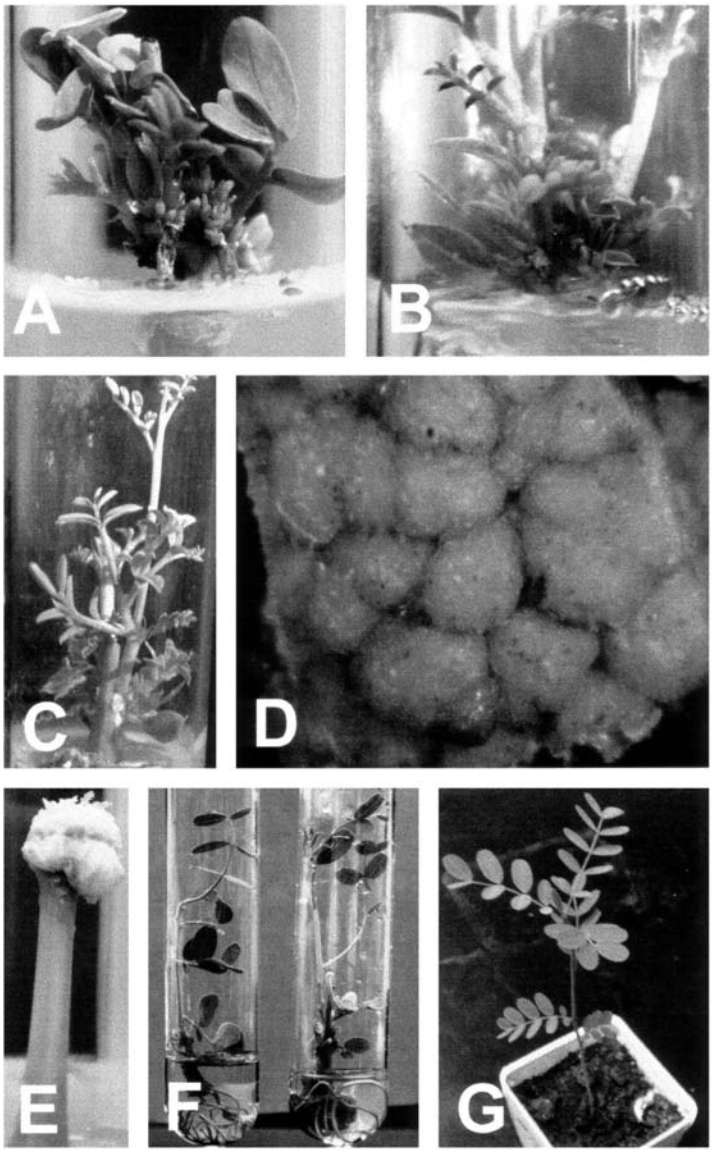

Fig. 1. Morphogentic response of *Sesbania drummondii* explants. *A* - Induction of multiple shoots from cotyledonary node on MS medium containing 22.2 μM BA; *B* - Stem node (from 3-month-old plant) showing enhanced branching on MS medium containing 2.27 μM TDZ; *C* - Proliferation of elongated shoots from axillary node on MS medium supplemented with 11.1 μM BA and 2.46 μM IBA; *D* - Cotyledon explant subcultured on MS medium with 2.27 μM TDZ showing proliferation of callus with embryoid-like structures; *E* - Hypocotyl explant cultured on 4.4 μM BA and 2.69 μM NAA showing callus on the apical part; *F* - Elongated shoots with roots (complete plantlets); *G* - Acclimatized *Sesbania drummondii* plant growing on peat moss.

per explant increased. The maximum number of shoots per CN explant was achieved using 22.2 μM of BA (Table 1 and Fig. 1*A*). Of the two explants, CN produced higher number of shoots per explant (< 8) and longer shoots were developed within 4 weeks. However, the percentage of cultures showing regeneration were comparable in both explants (Table 1). Increasing BA concentration above 13.3 μM though increased axillary bud proliferation in CN but resulted in reduced growth of shoots. In *Simmondsia chinensis* 20 μM BA induced multiple shoots in nearly 100 % of the nodal explants (Agrawal *et al.* 2002).

Stem segments excised from 3-month-old plants proliferated into multiple shoots on MS medium containing 2.27 and 4.54 μM TDZ. TDZ induced 5 - 6 shoots per explant (Fig. 1*B*) that were healthier than those regenerated on BA. But the frequency of the regenerants varied from 50 - 60 % at both concentrations of TDZ. The basal part of each stem proliferated into large callus mass, which could not differentiate further. TDZ-induced multiple shoot regeneration by bud break has been reported in a number of woody species (Murthy *et al.* 1998). TDZ has also been shown to promote differentiation at much lower concentrations, and shoot

regeneration occurs with efficiency comparable to or greater than that of other cytokinins (Malik and Saxena 1992). In this study regeneration of explants from three-month-old plants cultured on 2.27 or 4.54 μM TDZ was found to compare with the 10-d-old seedlings explants cultured on 13.3 - 22.2 μM BA (Table 1).

When axillary and cotyledonary node explants were cultured on MS medium containing combinations of 8.8 or 11.1 μM BA, and 0.24 - 2.46 μM IBA, cotyledonary node showed higher frequency of shoot regeneration than axillary node (Table 2). Cotyledonary node also produced larger shoots than the axillary node except at the concentration of 8.8 μM BA and 2.46 μM IBA. The best combination for cotyledonary node proliferation was 11.1 μM BA and 2.46 μM IBA (Table 2). The frequency of shoot regeneration was 30 - 90 % in cotyledonary node explants while 30 - 50 % in axillary branch node. Out of many regenerated shoots one or two shoots were dominantly elongated on these combinations of BA and IBA (Fig. 1*C*).

MS medium supplemented with different concentrations of BA (8.8 and 11.1 μM) and IAA (0.28 - 2.85 μM) induced regeneration of shoots (50 - 90 %) from both CN and AN explants (Table 3). Maximum number of shoots produced per explant was > 4 in CN whereas number of shoots per AN explant was ≤ 3. The average shoot length was between 1.0 - 4.0 cm for CN and 0.75 - 2.5 cm for AN (Table 3). BA (11.1 μM) in combination with IAA (2.85 μM) induced rooting at the base of regenerated shoots in about 10 % of cotyledonary node explants. This event in *Sesbania drummondii* is comparable to the results in *Sesbania aculeata* (Bansal and Pandey 1993). Differentiation of shoot and root simultaneously from primary explants in presence of BA and IBA hints towards some suboptimal function of somatic embryogenic pathway. Some of the epidermal cells surrounding axillary bud might be induced to form embryoids leading to regeneration of complete plants. Similar observations were also made in *Glycine wightii* (Pandey and Bansal 1992).

For multiplication, when nodes from *in vitro* elongated shoots were subcultured on TDZ containing medium, approximately 10 shoots proliferated from each explant in 4 weeks. Thus 100-fold multiplication could be achieved from each primary explant in a span of 9 - 10 weeks by regular subculturing.

Subculturing of callus on 2.27 μM TDZ resulted in a large callus mass containing a number of shiny, globular structures, which looked-like embryoids (Fig. 1*D*). These callus cultures were maintained for three months by regular

Table 2. Number, length and percentage of shoots from cotyledonary node (CN), axillary branch node (AN) explants of *S. drummondii* following 5 weeks of culture on different concentrations and combinations of BA and IBA. Each mean is based on three sets of replicates, each of which consisted of 12 culture tubes. *Means having different letters in superscript are significantly different from each other ($P < 0.05$) according to *ANOVA* and *post hoc* Tukey HSD multiple comparisons test.

BA [μM]	IBA [μM]	Shoot length [cm]		Number of shoots [explant^{-1}]		Regeneration [%]	
		CN	AN	CN	AN	CN	AN
8.8	0.24	1.0^{a*}	1.0^{a}	3.16^{a}	1.66^{a}	75	50
8.8	0.49	3.5^{b}	2.0^{b}	3.00^{b}	1.25^{a}	70	50
8.8	2.46	1.0^{a}	1.5^{a}	1.40^{c}	0.80^{b}	50	40
11.1	0.24	1.0^{a}	1.0^{a}	1.66^{c}	1.00^{b}	30	30
11.1	0.49	1.5^{b}	1.0^{a}	3.50^{a}	2.00^{c}	75	50
11.1	2.46	7.5^{c}	2.5^{c}	4.77^{d}	1.18^{a}	90	40

Table 3. Number, length and percentage of shoots from cotyledonary node (CN), axillary branch node (AN) explants of *S. drummondii* following 5 weeks of culture on different concentrations of BA and IAA. Each mean is based on three sets of replicates, each of which consisted of 12 culture tubes. *Means having different letters in superscript are significantly different from each other ($P < 0.05$) according to *ANOVA* and *post hoc* Tukey HSD multiple comparisons test.

BA [μM]	IAA [μM]	Shoot length [cm]		Number of shoots [explant^{-1}]		Regeneration [%]	
		CN	AN	CN	AN	CN	AN
8.8	0.28	1.0^{a*}	0.7^{a}	2.28^{a}	0.8^{a}	75	50
8.8	0.57	2.5^{b}	2.0^{b}	1.12^{b}	0.8^{a}	60	50
8.8	2.85	2.5^{b}	-	3.60^{c}	-	50	-
11.1	0.28	1.0^{a}	1.0^{a}	2.33^{a}	0.6^{a}	90	90
11.1	0.57	3.5^{c}	2.5^{c}	3.50^{b}	1.5^{b}	75	50
11.1	2.85	4.0^{d}	2.5^{c}	4.27^{c}	3.0^{c}	90	90

Table 4. Morphogenetic response of hypocotyl and epicotyl explants of *S. drummondii* grown on different concentrations of BA and NAA.

BA [μM]	NAA [μM]	IBA [μM]	Hypocotyl callus [%]	Epicotyl callus [%]
4.4	-	-	30	30
8.8	-	-	60	40
9.7	-	-	70	30
8.8	1.34	-	80	60
4.4	2.69	-	100	100
4.4	5.37	-	90	90
4.4	8.00	-	90	90
2.2	-	0.24	0	30
4.4	-	0.24	0	30
6.8	-	0.24	0	30
2.2	-	0.49	50	50
4.4	-	0.49	50	50
6.8	-	0.49	30	30

subculturing. However, various attempts to stimulate embryogenesis or regeneration could not be achieved. BA induced multiple shoot regeneration directly from cotyledon has been reported in *S. bispinosa* (Kapoor and Gupta 1986). In *S. grandiflora* rapid bud proliferation from cotyledonary explants has also been shown as a means of mass propagation (Detrez *et al*. 1994).

Hypocotyl and epicotyl explants from *S. drummondii* cultured on various combinations of NAA, BA and IBA produced moderate to profuse callusing on the MS medium supplemented with 8.8 μM BA + 1.34 μM NAA; 4.4 μM BA and different concentrations of NAA (1.34 - 8.0 μM) (Table 4.). Proliferation of callus was mostly seen at the top of hypocotyls (Fig. 1*E*). Callus from hypocotyl grown on the medium supplemented with NAA and BA was compact, and greenish. In *Spilanthes acmella*, BA and NAA combination induced multiple shoots from hypocotyl segments (Saritha *et al.* 2002). The hypocotyl and epicotyl explants grown on the MS medium supplemented with 2.2 - 6.81 μM BA and 0.24 - 0.49 μM IBA also produced fast growing callus. In *Sesbania aculeata*, hypocotyl explants were found to differentiate into shoots directly when cultured on NAA and BA combinations (Bansal and Pandey 1993). Also in *S. bispinosa* and *S. formosa*, hypocotyls directly differentiated into multiple shoots (Zhao *et al*. 1993). Studies on *S. drummondii* support the contention of Zhao *et al*. (1993) that tissue culture response in *Sesbania* species is largely variable and dependent on explant type and age.

Roots were visible within 5 - 10 d following transfer of elongated shoots to the rooting medium. After 2 - 3 weeks, plantlets developed primary and secondary root systems (Fig. 1*F*). Frequency of rhizogenesis was almost 100 %. Increasing concentration of IBA above 0.24 μM had no effect on the frequency of root regeneration. Shoots also produced roots when transferred to basal medium containing no plant growth regulators, but the rooting was higher with IBA (0.24 - 2.46 μM). Shoot elongation was also achieved on the rooting medium. However, faster shoot elongation was recorded on MS medium containing 1.14 μM IAA.

Survival of 60 % *in vitro* raised plantlets was recorded after acclimatization in room conditions (Fig. 1*G*). But only 30 % plantlets survived after 3 months in green-house condition. One of the reasons for high plant mortality may be improper relative humidity (RH) in greenhouse. Conditions for acclimatization are being optimized by alteration in light and RH conditions.

In conclusion, an efficient protocol for rapid propagation of *S. drummondii* has been developed using explants from seedlings as well as 3-month-old plants. The plant growth regulators such as BA and TDZ were found to be effective in promoting shoot regeneration and multiplication.

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