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BRIEF COMMUNICATION

Micropropagation of *Sesbania rostrata* from the cotyledonary node

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Abstract

Multiple shoots were induced from the cotyledonary nodes derived from seedling of *Sesbania rostrata* on Nitsch (1969; N) medium supplemented with various concentrations of benzyladenine (BA). 1 mg dm^{-3} BA proved to be the best, eliciting 5.8 ± 1.0 shoots per explant in 100 % cultures. The elongation of shoots was best at 2.0 mg dm^{-3} BA. The shoot proliferation capacity increased to 7.5 shoots per explant following transfer of explants to the fresh shoot multiplication medium (MS + 1.0 mg dm^{-3} BA), after an initial incubation of 30 d. To further enhance number of shoots per explant an alternative strategy of cultivation of mother explant on fresh shoot multiplication medium after excision of shoots was adopted. Following the repeated harvesting of shoots an average of 33 shoots per explant could be obtained. The *in vitro* regenerated shoots produced roots when transferred to half-strength MS medium supplemented with 3 % sucrose and 1 mg dm^{-3} IBA. The developed plantlets were planted in the soil and transferred to the field after an acclimatization period of 3 - 4 months. These plants produced flowers and fruits in the field and exhibited the development of prominent and more organized stem nodules as compared to the *in vivo* raised plants of the same age.

Additional key words: auxins, cytokinins, shoot proliferation, stem nodule, tissue culture.

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Sesbania rostrata (Bremek & Obrem.), a member of the family *Papilionaceae*, is an annual shrub exhibiting a symbiotic association with rhizobial strain ORS 571 (Syn. *Azorhizobium sesbania*), in the stem nodules. *S. rostrata* is a native of Africa and was introduced in India in 1980s. Since then it has gained immense importance due to its ability to assimilate both the soil and atmospheric nitrogen at a significantly high level (*ca*. 200 kg N_2 ha^{-1} in 50 d; Vlachova *et al.* 1987). Thereafter, it is widely used as a green manure in the fields. In addition, it binds soil and is resistant to water logging with a high fodder value (Kwon and Beevers 1992).

The tree legumes are known for their recalcitrant nature. The regeneration protocol of *Sesbania sesban* employing the mature tree derived explants has been described earlier (Jha *et al.* 2003/4). The present study was undertaken to standardize the regeneration protocol for *S. rostrata* employing seedling-derived explants.

Seeds of *S. rostrata* were procured from the Indian Agricultural Research Institute (IARI), New Delhi and Satellite Center of National Bureau of Plant Genetic Resources (NBPGR) at Amrawati, India. Seeds were treated with 0.1 % (v/v) Polysan (*Polypharm Ltd*, Mumbai, India) for 5 min followed by thorough washing in running tap water for half an hour. They were scarified with 80 % suplhuric acid for 30 min, followed by surface sterilization with freshly prepared chlorine water (prepared by dropping concentrated HCl on the crystals of potassium permagnate followed by passing the gas in water) for 30 min and then were rinsed with sterile distilled water for 4 - 5 times and inoculated on Knop medium containing 0.8 % (m/v) bacterio-logical grade agar (*Qualigens, Galxo Fine Chemicals*, Mumbai, India)

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Abbreviations: BA - 6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; MS - medium of Murashige and Skoog; N - medium of Nitsch; NAA - α-naphthaleneacetic acid; 2iP - 6(γ,γ-dimethylallyl) adenine.
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and 1 % (m/v) sucrose (*Daurala*, Meerut, India), under aseptic conditions. 12 to 15-d-old seedlings were used as the source of cotyledonary explants. The cotyledonary nodes (1 cm) were placed on Nitsch (1969; N) medium with 0.8 % agar, 3 % sucrose and different concentrations (0, 0.1, 0.5, 1.0, 1.5, 2.0, 3.0 or 4.0 mg dm^{-3}) of 6-benzyl-adenine (BA), kinetin (KIN), 6(γ,γ-dimethylallyl) adenine (2-iP), zeatin, indole-3-acetic acid (IAA), naphthalene acetic acid (NAA) or 2,4-dichlorophenoxy acetic acid (2,4 D) (*Sigma Chemical Co.*, St. Louis, USA). The pH of media was adjusted to 5.8 with 1 M HCl/1 M NaOH, before autoclaving. The cultures were incubated at temperature of 25 ± 2 °C, relative humidity of 55 ± 5 % and were exposed to a photoperiod of 16 h with irradiance of 17.76 μmol m^{-2} s^{-1} provided by cool daylight fluorescent tubes (40 W; *Crompton*, Mumbai, India).

For *in vitro* rhizogenesis 3 - 4 cm long *in vitro* regenerated shoots were implanted on full-strength, half-strength and quarter-strength Murashige and Skoog medium (1962; MS) supplemented with different concentrations (1, 2, 3 mg dm^{-3}) of IAA or IBA.

For hardening, 8 to 9-week-old rooted shoots were initially transferred to sterile *1*) sand:garden soil (1:1), *2*) garden soil:sand:vermiculite (1:1:1), and *3*) garden soil:sand:cow dung (1:1:1). These plantlets were initially irrigated with dilute inorganic salt solution of MS medium for 10 - 15 d followed by tap water. Four months after acclimatization under culture room conditions (25 ± 2 °C, 55 ± 5 % RH and 17.76 μmol m^{-2} s^{-1}), these were transferred to the field.

The response has been expressed in terms of percentage of responding explants, average number of shoots per explant and average length of shoots. A completely randomized block-design with two replications was used and the data were subjected to analysis of variance (*ANOVA*) by Fischer's least significant difference (P = 0.05). Data expressed as percentage response were arcsine transformed before analysis. For each treatment of a replicate experiment, at least 48 explants were cultured.

The seeds of *S. rostrata* are covered by a hard seed coat like other legumes. Various methods have been adopted for scarification of seeds, however, the acid scarification has been found to be beneficial for the seed germination in this species as also been reported for other leguminous trees such as *Sesbania grandiflora* (Khattar and Mohan Ram 1983), *Acacia auriculiformis* (Mittal *et al.* 1989). The scarified seeds started germinating within 3 - 4 d after inoculation on Knop's medium with the emergence of radical.

The cotyledonary nodal explants of 12 to 15-d-old seedlings swell and started differentiating multiple shoot buds directly within 15 - 20 d of inoculation on all the concentrations of BA, however, the average number of shoots formed per explant and the average shoot length varied considerably at different concentrations. A maximum response of 5.8 ± 1.0 shoots per explant with an average shoot length of 3.7 ± 3.0 cm was recorded on 1 mg dm^{-3} BA, after 52 d of cultivation (Fig. 1*A*,*B*). The elongation of shoots was best at 2.0 mg dm^{-3} BA with an average shoot length of 5.4 ± 2.1 cm (Table 1). Subsequent transfer of explants to fresh medium increased the shoot number to an average of 7.5 ± 1.2 shoots per explant, following the transfer of explants to the fresh shoot multiplication medium after 30 d (Table 2). Moderate to profuse callus developed at the cut ends of the explants at all the concentrations of BA. Explants left after excision of shoots were not discarded, instead they were transferred to fresh shoot multiplication medium and maintained for 60 d. The cultured mother explants exhibited the development of new shoots. In this way, the mother explants continued to develop shoots up to four passages, however, the maximum number of shoots were produced in the second passage. During the fourth passage the shoot proliferation was poor and the explants turned brown thereafter. Adopting this procedure of cultivation of mother explants, an average of 33 shoots could be obtained per explant (Table 2). Similar approach has been adopted earlier in other species like *Cornus florida* (Kavierappa *et al.* 1997) and *Syzygium cuminii* (Jain and Babbar 2000).

Table 1. Effect of BA on the caulogenic potential of cotyledonary nodal explants of *Sesbania rostrata* supplemented to N medium, after 52 d of inoculation. Values represent means ± standard deviation. Values followed by the same letter in each column are not significantly different (P = 0.05). Relative amount of calli ++ - moderate, +++ - profuse.

Conc. [mg dm^{-3}]	Responding explants [%]	Number of shoots [$explant^{-1}$]	Shoot length [cm]
0.0	$100a^{++}$	2.5 ± 1.2c	3.4 ± 2.4b
0.1	$100a^{++}$	3.2 ± 0.0b	5.1 ± 3.5a
0.5	$100a^{++}$	3.7 ± 1.0b	4.9 ± 3.8a
1.0	$100a^{++}$	5.8 ± 1.0a	3.7 ± 3.0b
2.0	$100a^{+++}$	5.4 ± 1.9a	5.4 ± 2.1a
3.0	$100a^{+++}$	5.2 ± 1.7a	3.4 ± 3.3b
4.0	$100a^{+++}$	3.5 ± 2.6a	1.6 ± 1.1c

Besides BA, the effect of other cytokinins (2iP, KIN and zeatin) were also tested. The addition of KIN significantly reduced the average number of shoots at all the concentrations tried. Moreover, the shoots were stunted and malformed (Fig. 1*C*). Such shoots turned brown within 10 - 15 d. Likewise, 2iP and zeatin proved inhibitory (data not shown).

Three auxins (2,4 D, IAA or NAA) were also tried to assess their morphogenic response on the cotyledonary explants. On 2,4 D supplemented media, yellow-green compact calli developed all over the surface of the

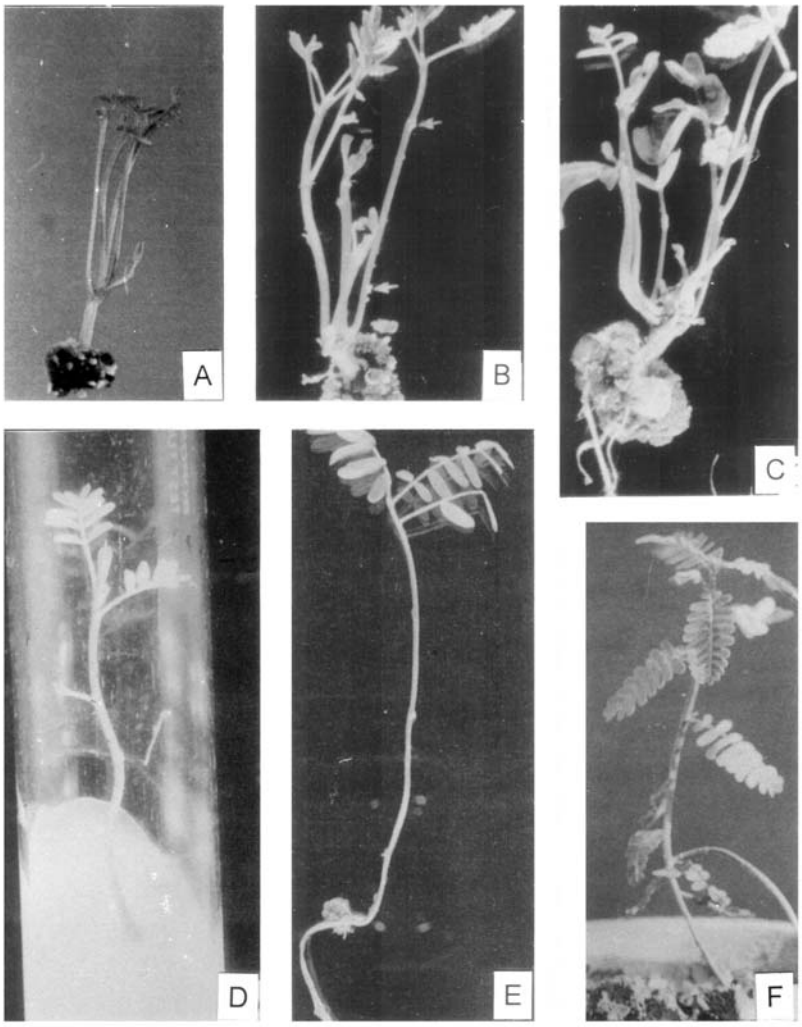

Fig. 1. *A*, *B*. Development of multiple shoots from the cotyledonary nodal explant with stem nodule initials (*arrow*) on N medium supplemented with 1 mg dm^{-3} BA, 15 (*A*) and 52 d (*B*) after inoculation. Compact nodular pale-yellow callus can be seen (×2.0 - *A*, ×2.5 - *B*). *C* - Development of malformed multiple shoots on KIN supplemented N medium (×2.5). *D* - The *in vitro* regenerated shoot on MS medium supplemented with 1 mg dm^{-3} IBA for induction of roots (×1.5). *E* - A rooted *in vitro* regenerated shoot prior to transfer (×1.6). *F* - 9-month-old *in vitro* raised plantlet of *Sesbania rostrata* in the field (×0.11).

explant, which however, failed to differentiate further even on prolonged culture. Likewise, on IAA or NAA supplemented media, the explants failed to elicit any morphogenic response.

2.0 - 4.0 cm long *in vitro* developed shoots were transferred to MS medium of different strength (*cf.* Materials and methods). Roots were initiated on all the three media, but in all three cases the root development was accompanied by development of profuse callus, with least in half-strength MS, therefore it was selected as optimum for rhizogenesis. To facilitate the development of strong root system, half-strength MS was supplemented with IAA or IBA (1 - 3 mg dm^{-3}). The best rhizogenic response was obtained on medium supplemented with 1 mg dm^{-3} IBA (Fig. 1*D*; Table 3).

The combination of garden soil and sand (1:1) supported the growth of transferred plantlets best (Fig. 1*E*). The plantlets were initially irrigated with dilute inorganic salt solution of N medium for 10 - 15 d followed by the tap water. The plantlets were covered by perforated polythene bags. After one month of acclimatization the plantlets were transferred to the field, where they grew well with development of prominent stem nodules (Fig. 1*F*) and later on these plants developed flowers and fruits. Vlachova *et al.* (1987) had earlier also reported the regeneration protocol for *S. rostrata*, employing cotyledon, hypocotyl and immature embryo, but observed a very low regeneration percentage. To conclude, the present report describes the efficient and reliable regeneration protocol of *S. rostrata* employing cotyledonary node *via* enhanced axillary bud break thereby eliminating the dependence on continued availability of seeds.

Table 2. Effect of passages on the shoot proliferation capacity of mother explant after repeated excision of developed shoots when maintained on shoot proliferation medium (N medium + 1 mg dm^{-3} BA). Values represent means ± standard deviation. Values followed by the same letter in each column are not significantly different ($P = 0.05$).

Passage number	Responding explants [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
Continuous culture	100a	5.6 ± 0.8b	4.7 ± 2.19a
Subcult. after 30 d	100a	7.5 ± 1.2a	5.5 ± 1.21a
1st passage	100a	8.2 ± 0.82a	5.2 ± 0.82a
2nd passage	100a	9.8 ± 1.1a	4.1 ± 1.3ab
3rd passage	91a	5.8 ± 2.1b	2.1 ± 0.81c
4th passage	62b	2.1 ± 1.9c	0.9 ± 1.34d

Table 3. Effect of different auxins supplemented to half-strength MS medium on the rooting of *in vitro* developed shoots of *Sesbania rostrata* after 30 d of inoculation. Values represent means ± standard deviation. Values followed by the same letter in each column are not significantly different ($P = 0.05$). Relative amount of calli ++ - moderate, +++ - profuse.

Auxin	Concentration [mg dm^{-3}]	Shoots developing roots [%]	Number of roots [shoot^{-1}]
Control	0.0	76d^{++}	5.6 ± 3.8c
IAA	1.0	96b^{++}	7.0 ± 3.5b
	2.0	94b^{+++}	8.3 ± 6.1a
	3.0	97a^{+++}	0.5 ± 4.4 e
IBA	1.0	100a^{++}	4.8 ± 3.0d
	2.0	100a^{++}	4.5 ± 2.7d
	3.0	80c^{+++}	5.4 ± 2.8c

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