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AXENIC PROPAGATION OF A WOODY LEGUME - PROSOPIS CINERARIA (L.) DUCE THROUGH COTYLEDONARY NODE EXPLANTS

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Abstract: Cotyledonary node explants of Prosopis cineraria (L.) Duce were excised from 10-day-old seedlings grown in vitro on Knop's medium [1] and cultured on Murashige & Skoog medium [2] supplemented with various cytokinins like N^6-benzyladenine;(6-dimethyl allylamine)-purine, kinetin or zeatin. Maximum number of shoots developed in 63% explants on 11µmol (2.5mg/l) N^6-benzyladenine adjuvanted MS medium after 60 d of culture. The adventive shoots differentiated in the axil of cotyledons. 89% shoots formed roots within 20-25d when subcultured on MS medium supplemented with 13.2µmol (3mg/l) indole-3-butyric acid. The plants developed in vitro were gradually transferred to the soil.

Keywords: Cotyledonary Node, Fabaceous Tree, Micropropagation, Prosopis Cineraria.

Abbreviations: AC, activated charcoal; B_5, Gamborg et al.'s medium; BA, N^6-benzyladenine; 2iP, (6-dimethyl allylamine)-purine; F.A.A., Formalin-acetic acid-alcohol; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; Kn, Kinetin; MS, Murashige & Skoog medium, NAA, α-Naphthalene acetic acid; NOA, β-Naphthoxyacetic acid.

Introduction

Micropropagation of trees is being increasingly recognised as a potential bypass technology for rapid afforestation to achieve enhanced biomass production. In addition, it offers means not only to conserve elite and rare tree germplasm but also for bringing about their genetic improvement. Prosopis is an important multipurpose fabaceous tree of arid tropics and has several species of value, predominant in the Thar desert. Though considered as a problematic and recalcitrant species to in vitro regeneration, during the past few years, a few reports with limited success have appeared employing both juvenile and mature explants [3,4,5].
The present investigations were undertaken to develop a protocol for micropropagation of a fabaceous taxon Prosopis cineraria, which is one of the two tree species that serve as lifeline for inhabitants of drier regions of Rajasthan in India.

Material and Methods

Seeds of Prosopis cineraria belonging to subfamily Mimosoideae were obtained from the Central Arid Zone Research Institute, Jodhpur, India. They were scarified and soaked in distilled water for 8hr at room temperature and surface sterilized with freshly prepared chlorine water (3.5±0.5mg/l) for 45min. Chlorine was obtained by addition of 30ml of concentrated hydrochloric acid to 4g of potassium permanganate. The chlorine water was prepared by bubbling chlorine in 500ml of distilled water for 15-20min. Thereafter, the seeds were washed three times with sterilized distilled water and germinated aseptically on Knop's medium containing 2% sucrose (British Drug House, Poole, England) and 0.8% agar (Hi-media, Mumbai, India). The pH of media was set at 5.8 before autoclaving at 121ºC at 1.06 kg/cm^2 pressure for 15min.

About 0.8-1cm long segments of cotyledonary node were excised from 10-day-old seedlings and cultured on MS basal medium alone as well as adjuvanted with cytokinins (BA, Kn, 2iP or zeatin) at 0.5 to 3mg/l levels. The media contained 3% sucrose and 0.8% agar. Cultures were maintained at 25±2ºC and 55±10% relative humidity under white fluorescent light at 40µ cEm^{-2}s^{-1} irradiance emitted by 40W Crompton incandescent tubes programmed for 16 hr photoperiod. Explants were subcultured on a fresh medium after every 25-30 days and data were recorded at an interval of 10-15d. The final data were scored after 60d of culture. The in vitro reared shoots were excised and reared on MS medium supplemented with 1-3mg/l auxins (IAA, IBA, NAA or NOA) for root induction. A minimum of 30 replicates were raised for each treatment and all experiments were repeated at least once. The average number of shoots per responding explant has been represented as mean value indicating the standard deviation (mean±S.D).

For histological studies, tissues were fixed in F.A.A. and dehydrated through a graded ethanol-xylene series, followed by infiltration and embedding in paraffin wax. Serial sections, 20-25µm thick, were cut with a rotary microtome and affixed to slides. These were dewaxed, stained with safranin-fast green combinations, dehydrated and mounted in canada balsam. Photomicrographs were taken by a 35mm camera attached to the Leitz Orthoplan Universal Microscope.

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Results and Discussion

Germination: Seeds of Prosopis are hard to germinate [6]. To enhance germination various pre-treatments were given viz. (i) scarification, (ii) soaking in distilled water for 4-12hr at room temperature, (iii) scarification followed by soaking in distilled water for 4-12hr and (iv) concentrated sulphuric acid treatment for 5-20 min. good germination (100%) was achieved in seeds which were scarified and soaked in distilled water for 8hr. Hence, this pre-treatment was routinely employed for all subsequent experiments.

Morphogenic response: The cotyledonary nodes and shoot tips readily differentiated multiple shoots on medium adjuvanted with various cytokinins. The other explants viz. epicotyl, cotyledons and hypocotyl formed only callus along with a few sporadic roots. The results pertaining to the experiments conducted only on cotyledonary nodes are presented in this communication.

Differentiation of shoots: Of the various basal media used, MS was most suitable for differentiation of shoots in juvenile explants of Prosopis cineraria, as also reported earlier for its mature explants [5,7,8]. Within 10-15d of inoculation, adventive shoot buds developed on cotyledonary explants on MS medium containing different cytokinins viz. BA, Kn, 2iP and zeatin. Optimum growth of multiple shoots was observed on BA supplemented media (Figs 1B, C; [9]) as compared to control (MS basal medium; Fig.1A) as well as other cytokinins in which only one or two shoots per explant were organised. When cultured on BA (2.2-13.2µmol or 0.5-3mg/l) supplemented medium, the number of shoots per explant steadily increased with increasing levels of BA till 11µmol (2.5 mg/l) level, thereafter the response decreased (Table 1).

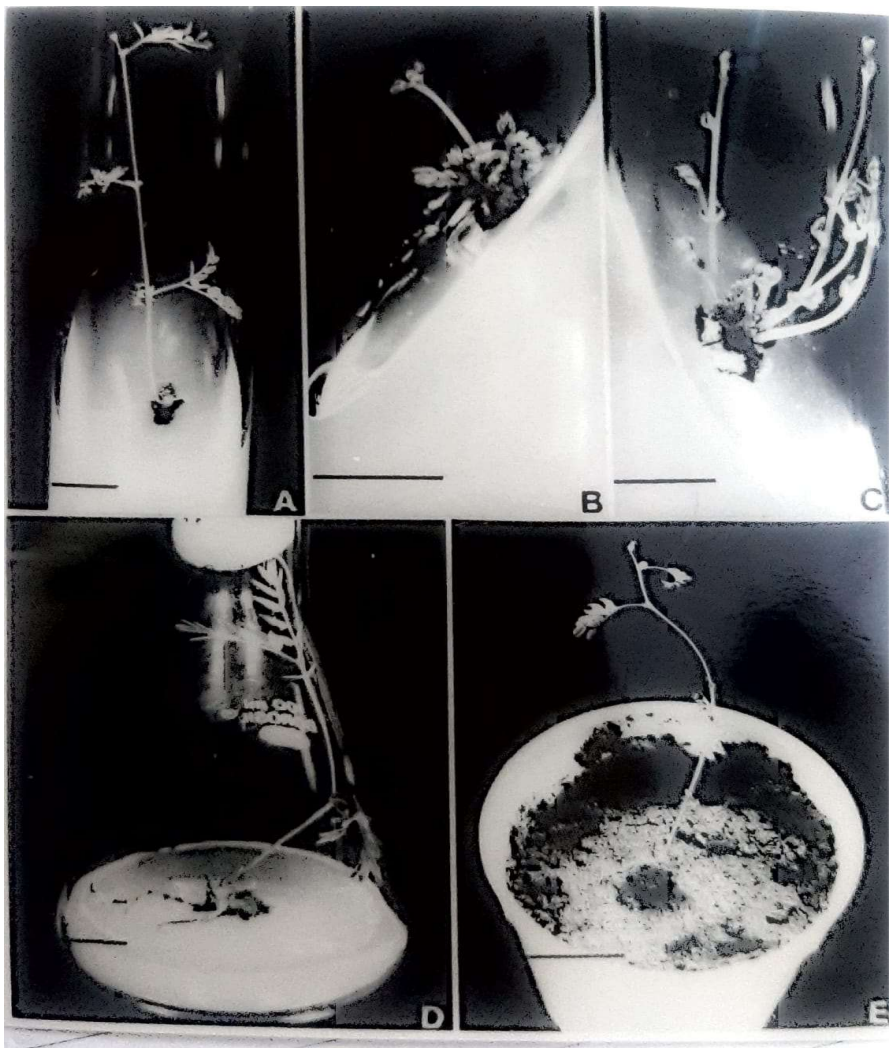

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Fig.1. (A-E). *In vitro* morphogenic response of *P. cineraria* cotyledonary node explants. **(A)** Single shoot developed on MS basal medium after 60d of culture.x 1.2 **(B,C,)** Differentiation of multiple shoots on 2.5mg/l BA adjuvanted medium. B x 2, C x 1.6 **(D)** *In vitro* raised shoots developing roots on 3mg/l IBA supplemented medium x 1.1 **(E)** *In vitro* raised plantlets growing on a mixture of sand and soil (1:1)

Table 1. Morphogenic response of Prosopis cineraria cotyledonary node explants on MS medium supplemented with BA, grown for 60d.

BA (mg/l)	Number of explants	Explants forming shoots (%)	Average number of shoots per explant	Shoot length (cm)
0	56	100(+) *	2.2±0.8	0.2-1.5
0.5	59	100(+)	3.5±1.5	0.2-2.5
1.0	54	100(+)	4.0±1.8	0.5-5.5
1.5	63	100(+)	5.0±2.2	0.2-5
2.0	60	100(++)	4.9±2.6	0.2-7.5
2.5	63	100(++)	9.1±4.0	0.2-8
3.0	60	100(++)	6.7±3.8	0.2-8

*Relative amount of callus: (+) = little, (++) = moderate.

The number of shoots varied from 2 to 15 and a maximum average of 9.1 shoots per explant was reached at 11µmol (2.5mg/l) BA supplemented MS medium, within 60d of incubation. The shoots were healthy and achieved a length up to 8cm. Optimum response was thus obtained at 11µmol (2.5mg/l) BA containing MS medium. However, some reports [8,10] show kinetin to be better for shoot formation in axillary bud explants of mature trees. On the other hand, a combination of BA (cytokinin) and NAA (auxin) has been found to be essential for shoot differentiation [3,11,12]. Multiple shoots have also been regenerated successfully on MS medium supplemented with cytokinin and auxin in combination [5,12,14]. However, during the present investigations, it has been found that BA alone is sufficient for multiple shoot induction as also reported earlier [9].

During the present investigations, explants also formed small amounts of friable to compact calluses at their cut ends as well as on their surface, which turned brown within 7-10d. Subsequently, shoots regenerated directly in the axil of cotyledons in 10-15d of culture. In contrast to the earlier report [10], wherein only 3-5 shoots developed in a single axillary bud explant in 100-110d, in the present investigation, an average of about 9 shoots per cotyledonary node explant were formed within 60d. Thus, not only the incubation time has been reduced to nearly half but also the yield has been doubled.

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Origin of accessory shoot buds: Similar to our earlier investigations on Acacia nilotica subsp. indica [13] longitudinal sections of the cotyledonary node explants of P. cineraria (control) showed absence of any axillary bud or meristematic tissue at the time of inoculation (Fig.2A). However, accessory multiple shoot buds developed on BA supplemented medium within 10-15d of culture. Each shoot bud showed a distinct vasculature indicating its de novo origin (Fig. 2B), thus showing that incubation on BA adjuvanted medium induced meristematic zones which differentiated multiple shoot buds.

Rooting of excised shoots and acclimatization of plantlets: Prosopis species are generally known to be rather difficult to root under axenic cultures. Experiments carried out with cuttings derived from adult stem and seedings in P. tamarugo failed to root [14]. During the present experiments with P. cineraria, about 50-60 d old in vitro grown shoots varying from 1-8cms in length, were cultured on MS medium containing IAA, IBA, NAA or NOA at 1-3mg/l levels. Within 20-25d, small roots were observed at the base of shoots in different auxin supplemented MS media (Fig. 1D). While IAA, NAA and NOA elicited a very poor response, rooting was accomplished at 14.7µmol (3mg/l) IBA supplemented MS medium in 89% explants (Table 2) as also reported earlier [5,8]. Similarly, 65% rooting has been reported in in vitro raised P. cineraria shoots when treated with a very high concentration of IBA (492µmol or 100mg/l) on MS medium (½ strength) for 4hr, followed by transfer to MS medium (½ strength) containing 5000mg/l AC [4]. According to them, incubation in dark for 5d at 33±2ºC was beneficial for early root induction.

Transfer of about 7-10d old P. cineraria shoots to the rooting medium (MS + 14.83µmol or 3mg/l NOA) was found to be essential to prevent defoliation [15]. However, in the present experiments even after prolonged periods of culture, defoliation did not occur. The in vitro raised plantlets were successfully transferred to small pots containing garden soil and sand (1:1, Fig. 1E).

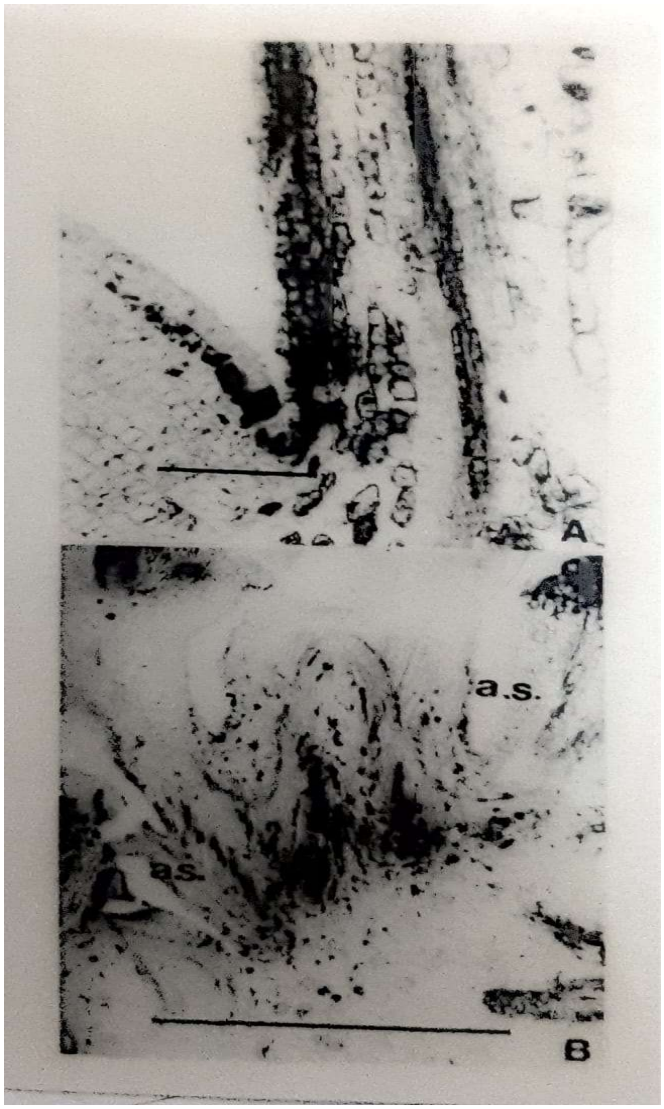

Fig.2. (A,B) Photomicrographs of *P.cineraria* cotyledonary node longitudinal sections. **(A)** Explant at the time of inoculation, without axillary bud or meristem x 2 **(B)** Same, showing adventive shoots (a.s.) developed in the axil of a cotyledon in the nodal region, after 45d of culture on MS + 2.5mg/l BA medium x 4.8

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Table 2. Rhizogenic response of P. cineraria shoots, reared on MS medium adjuvanted with various auxins, after 30d.

Auxin (mg/l)	Number of shoots	Shoots forming roots (%)
0 (control)	24	0
IAA		
1	20	0
2	22	7.5
3	26	11.1
IBA		
1	22	18.1
2	24	8.3
3	24	89.1
NAA		
1	24	8.3
2	20	10
3	25	4
NOA		
1	24	8.3
2	20	10
3	25	4

Conclusion

A successful protocol has been developed for rapid multiplication of Prosopis cineraria through cotyledonary node explants.

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