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Micropropagation of an Arid Zone Leguminous Tree – *Acacia Senegal* (L.) Willd Via Callus

Parul Gupta

Department of Botany, BBD Government College, Shahpura, Jaipur, Rajasthan- 303130, India.

Abstract

An efficient micropropagation system was developed for an economically important arid zone tree species-*Acacia senegal* (L.) Willd., commonly known as Gum Arabic. Callus induction and high frequency regeneration was obtained on Murashige and Skoog (MS) medium along with auxins, (NAA, 2,4-D, IAA), cytokinins (BAP, Kn) and additives like AA and AS. Rooting of the regenerated shoots was initiated on ½ MS-medium + NAA (2.5 mgl^{-1}) + IBA (2.5 mgl^{-1}) and low sucrose level (3%). However, further elongation and development of roots was achieved on ¼ MS + IBA (0.5 mgl^{-1}) and still lower sucrose level (1%). For the hardening and acclimatization , the *in vitro* regenerated plantlets were transferred to a sterile vermiculite and sand mixture in a 3:1 ratio. Subsequently the hardened plantlets were transferred to a green house and finally to the fields.

Keywords: Micropropagation, *Acacia senegal*, Callus, differentiation.

Abbreviations : MS : Murashige and Skoog's medium, AA : Ascorbic acid, AS: Adenine sulphate, NAA:1-Napthalene acetic acid, IBA: 3-Indole Butyric acid, 2,4-D: 2,4–Di chlorophenoxy acetic acid, BAP: 6–Benzyl amino purine.

Introduction

Acacia senegal, commonly called as 'Kumbat', is an important leguminous tree used for fuel, fodder, tannins, gums and timber. The tree enriches the soil mineral content through symbiosis with *Rhizobium* and mycorrhiza (Colonna *et al.,* 1990). The works of Badji *et al.* (1991) and Danthu *et al.* (1992) demonstrated the feasibility of producing horticultural cuttings of *Acacia senegal*, as well as its limitations. The application of tissue culture to tree improvement offers opportunities for increasing forest productivity (Sommer and Brown, 1979). The morphogenetic response of seedling explants of *A. senegal* was reported for first time be Dave *et al.* (1980). This and subsequent attempts (Hustache *et al.,* 1986, Badji *et al.,* 1993) showed that the material responded to exogenous stimuli. Rapid *in vitro* propagation via mature nodal explant (Gupta *et al.,* 1994, Kaur *et al.*, 1998) and via cotyledonary node explant (Kaur *et al.,* 1996) have been reported. Adventitious regeneration directly from organs generally gives a limited number of propagules. This number can be increased several folds by an intervening callus phase (Hussey, 1983). Moreover, callus cultures offers many advantages for isolation of mutants / somaclonal variants in higher plants. This paper describes the procedure employed to develop an uniform, reproducible and regenerative system using callus cultures of *A. senegal.* To the best of our knowledge, this is the first report on complete plant regeneration from callus of *A. senegal.*

Materials and Methods

For callus proliferation, the explants used were obtained from aseptically raised seedlings. Seeds of *A. senegal* were procured from Central Arid Zone Research Institute, CAZRI, Jodhpur as well as collected seasonally from the experimentally marked trees. Seeds were washed thoroughly with 2% extran detergent and then with sterile distilled water to remove every trace of detergent. Subsequently they were surface sterilized with 0.2% mercuric chloride solution for 5-7 minutes, again thoroughly washed in sterile distilled water and planted on sugar agar medium with 1.0 mgl^{-1} BAP. Seeds germinated after 7-10 days. Their germination was also tried on simple agar medium with 3% sucrose and without BAP but seedlings so obtained were not very sturdy and were weak and elongated. Cotyledons and the hypocotyl segments of seedlings were subsequently inoculated on MS-Medium enriched with various combinations and concentrations of auxins and cytokinins along with additives like AA and AS . The callus obtained was regularly subcultured at 6 weeks interval and maintained on MS–medium supplemented with 2,4-D (2.5 mgl^{-1}) + BAP (0.5 mgl^{-1}) + AA (10.0 mgl^{-1})

For differentiation, the callus tissues were transferred to MS-medium enriched with various combinations and higher concentration of cytokinins along with a lower concentrations of auxin. Cultures were regularly subcultured and regular observations were made. Shoots differentiated from the callus were isolated and transferred to the experimental rooting media . Phytagel was used at concentration of 2.0 gl^{-1} for gelling the rooting medium. Various combinations and concentrations of auxins (IBA, IAA, NAA) were tried for rooting and their solutions were used to give pulse treatment prior to their placement on the rooting medium.

The cultures were maintained at a temperature of 30±2° and 40-70% relative humidity in the culture room. A daily regime of 16 hours light followed by 8 hours dark was given to the cultures. Light was provided in a combination of cool white fluorescent tubes and incandescent bulbs in the ratio 3:1. The light intensity as irradiance was varied from 1000 lux to 4000 lux. The temperature, light and relative humidity conditions were varied according to the experiments to get optimal regeneration.

All the experiments were repeated thrice and six replicates per treatment were taken. Suitable control was maintained with each experiment. Regular subculture was done when required. Observations were taken regularly and all the data has been analysed statistically using student's t– statistics at 5% level of significance meaning 95% confidence limit.

Results and Discussion

Callus induction was observed in all types of explants viz. hypocotyl, cotyledons, leaflets, rachis and stem explants. However, the hypcotyl and cotyledon were found best for healthy and vigorous growth of callus. Various auxins, cytokinins and additives showed varied response. It was observed that 2,4-D (2.5 mgl^{-1}) along with BAP (0.5 mgl^{-1}) AA (10 mgl^{-1}) and AS (25 mgl^{-1}) was best for callus induction (Table 1). The callus so produced was compact and green in colour. It grew profusely and possessed high regeneration potential (Fig. 1A) whereas callus derived on medium supplemented with 2, 4-D alone failed to differentiate fully. Subsequent induction of shoot bud organogenesis and plantlet

regeneration involved transferring callus to shoot bud induction medium containing different concentrations of BAP and Kn along with auxins. It was observed that neither BAP nor Kn alone proved beneficial in callus organogenesis. However, optimum level of shoot bud formation was recorded in shoot bud induction media containing BAP (2.5 mgl^{-1}) and Kn (2.5 mgl^{-1}) along with AS (2.5 mgl^{-1}) AA (10.0 mgl^{-1}) and glutamine (146.0 mgl^{-1}) (Table-2). The entire piece of callus tissue turned into a mass with globular structures which later developed into shoots (Fig. 1B). Further elongation and development of shoot buds into shoots was achieved on MS-medium fortified with BAP (0.5 mgl^{-1}) + Kn (0.5 mgl^{-1}) and same additives as in shoot bud induction media (Fig. 1C).

Among the various rooting media tested for rooting of *in vitro* regenerated shoots, the best for inducing rooting was ½ MS + IBA (2.5 mgl^{-1}) + NAA (2.5 mgl^{-1}) + AS (25.0 mgl^{-1}) Fig. 1D. However, further elongation of roots took place on ¼ MS + IBA (0.5 mgl^{-1}) and sucrose (1%) (Fig. 1E) . Temperature and humidity also played an important role in root quality. Upto 60% rooting was observed at 32-34°C and 70-80% relative humidity. An initial dark period of 24-36 hours was essential for root induction. The *in vitro* regenerated plantlets are thus ready for hardening and acclimatization before transfer to the fields. Hardening was done on sterile vermiculite and sand mixture in ratio (3:1) (Fig. 1F) and kept under controlled environmental conditions of high relative humidity and temperature. But only 40-50% success rate was observed, mortality rate was quite high.

Thus, from the on-going account, it is evident that a protocol has been standardized for rapid *in vitro* regeneration of *A. senegal* plantlets. However, scaling up of hardening and acclimatization is needed for mass propagation of an elite tree of *A. senegal* or perhaps allied species. This plant meets all the requirements of social forestry and the demand of plantlets can be met with the plants regenerated through tissue culture.

Table 1 : Effect of auxins on callus induction

Medium	:	MS + Sucrose 3% + BAP (0.5 mgl^{-1}) + Auxins viz. 2, 4-D, NAA, IAA.
Inoculum	:	Hypocotyl and cotyledons of seedlings grown *in vitro*.
Incubation	:	At 30 ± 2°C in 16 h photoperiod (2500-3000) for 4 wks.

S.No.	Auxins treatment		Response	Remarks
1.	Control, MS + ascorbic acid (10.0 mgl^{-1})		-	Callus usually greenish
2.	2,4-D	0.5 mgl^{-1}	+	Callus usually greenish slightly compact fast growing and fragile.
		1.0 mgl^{-1}	+	
		2.0 mgl^{-1}	++	
		2.5 mgl^{-1}	+++	
		3.0 mgl^{-1}	+++	
		5-10 mgl^{-1}	+++	

3.	NAA 0.5 mgl^{-1} 1.0 mgl^{-1} 2.0 mgl^{-1} 2.5 mgl^{-1} 3.0 mgl^{-1} 5-10 mgl^{-1}	- + ++ ++ ++ +++	Callus is cream in colour and very compact. Slow growth after 3-4 days.
4.	IAA 0.5 mgl^{-1} 1.0 mgl^{-1} 2.0 mgl^{-1} 2.5 mgl^{-1} 3.0 mgl^{-1} 5-10 mgl^{-1}	- - + + ++ ++	Compact callus brownish cream in colour. Poor growth of callus.

- no callus   + moderate callus
++ good callus   +++ Profuse callus

Table 2: Effect of cytokinins and auxin on the morphogenetic responses of *A. senegal* calli

Medium	:	MS + Sucrose (3%) + AA (10.0 mgl^{-1}) + AS (25.0 mgl^{-1}) + BAP/Kn (0.5-4.0 mgl^{-1}) + IAA/NAA/2,4-D (1.0-3.0 mgl^{-1}) + Glutamine (146.0 mgl^{-1})
Inoculum	:	Callus derived from hypocotyl and cotyledons (~ 600 mg fresh weight).
Incubation	:	At 32 ± 2°C in 16 h photoperiod (2500-3000 ix) for 4-5 wks.

S.No.	Treatment	No. of shoot buds induced tissue *Mean± $t_{0.05}$ S.E. (x)	No. of shoots developed per unit callus tissue *Mean±$t_{0.05}$ S.E. (x)
1.	**BAP (4.0 mgl^{-1}) + NAA**		
	0.1	6.0±1.47	2.17±0.79
	0.5	3.0±0.93	1.5±0.57
	1.0	1.67±0.79	0.67±0.55
	2.0	0.5±0.57	
2.	**BAP (4.0 mgl^{-1}) + IAA**		
	0.1	2.33±1.08	0.83±0.43
	0.5	2.0±0.99	0.66±0.55
	1.0	0.5±0.57	-

	2.0	0.33±0.54	-
3.	**BAP (4.0 mgl^{-1}) + 2,4-D**		
	0.1	4.67±1.27	1.67±0.85
	0.5	2.5±1.10	1.33±0.55
	1.0	1.0±0.66	0.33±0.55
4.	**BAP + Kn + NAA**		
	0.5 0.5 0.1	-	-
	1.5 1.5 0.1	6.5±1.10	5.5±1.10
	2.0 2.0 0.1	7.67±1.58	2.5±1.10
	2.5 2.5 0.1	10.5±1.43	2.5±1.10
	3.0 3.0 0.1	8.67±0.87	1.67±0.87

* Values are 95% confidence limits for mean

Figure 1: Plantlet regeneration of *A. senegal* via callus. (A) Callus induction and growth on MS medium supplemented with 2,4-D (2.5 mgl^{-1}) + BAP (0.5 mgl^{-1}) + AA (10.0 mgl^{-1}); (B) Shoot differentiation from callus on MS medium with BAP (2.5 mgl^{-1}) + Kn (2.5 mgl^{-1}) along with AS (25.0 mgl^{-1}), AA (10.0 mgl^{-1}) and glutamine (146.0 mgl^{-1}) (C) Fully developed shoots ready for rooting (D) Initiation of roots in cultured shoots in ½ MS + IBA (2.5 mgl^{-1}) + IBA (2.5 mgl^{-1}) + NAA (2.5 mgl^{-1}). (E) Elongation of roots in ¼ MS + IBA (0.5 mgl^{-1}) + 1% sucrose. (F) *In vitro* regenerated plantlet in sterile vermiculite sand mixture (3:1) for hardening and acclimatization.

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