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Regeneration of a Multipurpose Woody Legume - *Acacia auriculiformis* through Mature Tree Nodal Explants

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Abstract

Multiple shoots have been induced in nodal explants of 30-year-old *Acacia auriculiformis* trees on Nitsch's medium (N medium) supplemented with 1.0 mg/l N_6-benzyladenine. Addition of 0.1 mg/l adenine sulfate to N medium containing BA, enhanced the caulogenic response whereby the number of shoots developing on each node increased from three to fifteen. Plantlets were obtained by rooting axenically raised shoots on N medium (half strength) with 1.0 mg/l naphthalene acetic acid. Use of activated charcoal (0.01%) in the medium completely suppressed callus formation at the root-shoot junction.

Introduction

Importance of tree legumes to humanity is well documented. Despite their recalcitrant behaviour in culture, keen interest in application of *in vitro* techniques for rapid micropropagation of woody species in recent years, clearly indicates their importance.

A. auriculiformis A. Cunn. ex Benth., is an important tree legume. It is of medicinal importance because of its spermicidal and anti-HIV properties along with its safe use on vaginal epithelium (Girijashankar 2011). *A. auriculiformis* is grown for timber and used as fuel. As a popular avenue tree, adapted to a wide range of soil types and climatic conditions, it is a potential candidate for afforestation programs. Its rapid early growth, ability to fix nitrogen, and tolerance of infertile, acidic, alkaline, saline and seasonally waterlogged soils make it a very useful species for the rehabilitation of degraded lands. In addition, this tree can tolerate a moderate dry season (Azad et al. 2011). Most of the available research on the regeneration of heartwood species within the *Acacia* genus focuses on juvenile explants. These materials include the cotyledonary bud of *A. albida* (Ranga Rao and Prasad 1991) and its root (Ahée and Duhoux 1994), the shoot apex of *A. koa* (Skolmen and Mapes 1976), the dissected embryo of *A. melanoxylon* (Jones 1986), and

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the cotyledonary node of both *A. nilotica* and *A. tortilis* (Dewan et al. 1992), (Nangia and Singh 1996). However, plantlets so formed may not necessarily perpetuate the desired superior qualities of the parents. Therefore, regeneration of tissues excised from old trees is most desirable, as the genotype of offsprings is predictable. This has a distinct advantage, especially in tree improvement programs (Bonga and Durzan 1987). Hence, the present studies were undertaken to define optimal culture conditions for cloning of 30-year-old *A. auriculiformis* trees growing luxuriantly. Protocols for plantlet regeneration from seedling-derived explants, *i.e.* axillary buds (Mittal et al. 1989) and shoot tips (Ranga Rao and Prasad 1991) of this species have been reported. A review mentions detailed survey of research carried out on *in vitro* propagation of different species of *Acacia via* direct organogenesis has been published (Gantait et al. 2018). Stem nodes of 18-month old plants of *A. auriculiformis* have been reported (Girijashankar 2011).

Materials and Methods

Twigs were collected from mature trees (30-year-old) growing on Kamla Nehru Ridge, near the University of Delhi. After removing phyllodes, the material was treated with dilute detergent solution (5% Polysan, Polypharma Pvt. Ltd., Mumbai) for 30 min. and kept under running tap water for 20 min. Subsequently, they were immersed in 0.5% solution of a fungicide (Bavistin, Delhi) for 20 min. Thoroughly washed for one min. with hot water (58°C), the material was surface sterilized with 0.2% (w/v) mercuric chloride (Qualigens, Mumbai) for 15 minutes. This was followed by vigorous shaking in rectified spirit for one minute. Subsequently, all traces of sterilant were removed by 3 or 4 thorough washings with sterile distilled water. Twigs were then cut into 1-1.5 cm long uni-nodal explants in the laminar hood. Culture media and culture conditions: For caulogenesis, cultures were reared on N medium (Nitsch and Nitsch 1969) alone or supplemented individually with cytokinins, *viz*. BA, Kn, 2iP or zeatin. Optimum concentration of cytokinin (1 mg/l BA) was combined with different auxins (IAA, IBA or NAA), GA and Ad.S. as adjuvants. Experiments included in Table 1, were conducted in February and repeated in March and December months. Those in Table 2, were conducted in January and repeated in April. While those in Tables 3 and 4 were performed in April and repeated in May. Others included in Table 5, were conducted in May and repeated in June.

As a source of carbon, 3% sucrose (Daurala, Delhi) was added to the medium. Gelling was done with 0.8% agar (Qualigens, Mumbai). For inducing rhizogenesis, axenically raised shoots were transferred to MS and N (Nitsch and Nitsch 1969) media (full and half-strengths) alone or adjuvanted individually with different auxins like IAA, IBA, NAA and NOA. AC was added in the range of 0.01-0.05% to the rooting medium to suppress callusing. In addition, shoots were also subcultured for rooting on Knop's medium (Knop 1865). All the chemicals used for media preparation were of analytical grade (Qualigens, Merck and Sigma-Aldrich). The *p*H of the media was adjusted to 5.8

using 1N NaOH or 1 N HCl, before autoclaving at 121°C for 15 min. at 1.06 kg/cm^2 pressure.

Cultures were raised on medium dispensed in 2.5cm x 15 cm test tubes (Borosil, India) which were maintained at 25 ± 2°C with 55 ± 5% relative humidity and 16 hr photoperiod under 450-640 μW/cm^2 light provided by 40W Philips incandescent tubes in culture room. The explants were subcultured at an interval of 30 days, wherever necessary. Observations were recorded at an interval of 12-15 days. The final data were recorded after 60-65 days of growth *in vitro*. The morphogenic responses were photographed using Leica Aristofort system.

Results and Discussion

Nodal explants, reared on N medium alone and supplemented with 0.1-3.0 mg/l of individual cytokinins (BA, Kn, 2iP and zeatin) showed bud-break within 7-10 days. All the cytokinins tried, supported multiple bud induction. However, 1 mg/l BA was most effective with maximum average of 3.15 ± 1.58 shoots per explant (Table 1).

Table 1. Morphogenic response of 30-year-old *A. auriculiformis* nodal explants on BA, after 65 days of culture. Experiment was conducted in February and repeated in March and December months.

BA (mg/l)	Number of explants	Explants forming callus (%)	Degree of callus formation	Explants forming shoots (%)	Average number of shoots per explant	Average shoot length (cm)
0.0	36	55	+*	58^{a}**	2.04 ± 0.94	0.55
0.1	42	33	+	62^{a}	1.88 ± 0.80	1.05
0.5	39	36	+	67^{a}	1.53 ± 0.92	0.85
1.0	38	42	+	84^{a}	3.15 ± 1.58	1.30
2.0	35	63	++	83^{a}	2.51 ± 0.93	1.05
3.0	31	68	++	64^{a}	1.90 ± 0.94	1.10

*Relative amount of callus: (+) little, (++) good, (+++) profuse. ** Values in a column followed by the same superscript are not significantly different as determined by Chi-square test at 5% level of significance.

Shoots formed were stout, straight and healthy with well-developed phyllodes (Fig. 1A). The regeneration potential of explants on Kn supplemented medium was very poor. In fact, higher levels had inhibitory effect on percentage of responding cultures, shoot number as well as shoot length (Table 2).

Table 2. Response of *A. auriculiformis* nodal explants to Kn supplemented N medium. Data recorded after 65 days of inoculation. Experiment was conducted in January and repeated in April.

Kn (mg/l)	Number of explants	Explants forming callus (%)	Degree of callus formation	Explants forming shoots (%)	Average number of shoots per explant	Average mean shoot length (cm)
0	42	38	+*	33[b]**	2.04 ± 0.94	0.55
0.1	40	50	+	70[a]	1.88 ± 0.80	1.05
0.5	46	65	+	52[a,c]	1.53 ± 0.92	0.85
1.0	36	36	+	25[b,c]	3.15 ± 1.58	1.10
2.0	36	69	++	53[a,c]	2.51 ± 0.93	1.05
3.0	44	54	+	41[a,b,c]	1.90 ± 0.94	1.10

*Relative amounts of callus: (+) little, (++) good, (+++) profuse. ** Values in a column followed by the same superscript are not significantly different as determined by Chi-square test at 5% level of significance.

Shoots developed per explant at levels higher to 0.1 mg/l Kn. and phyllodes remained under-developed (Fig. 1B). In medium supplemented with 2iP, more than two shoots differentiated per explant across various concentrations tested (Table 3).

Table 3. Effect of 2iP on nodal explants of *A. auriculiformis*, reared on N medium for 65 days. Experiment was performed in April and repeated in May.

2iP (mg/l)	Number of explants	Explants forming callus (%)	Degree of callus formation	Explants forming shoots (%)	Average number of shoots per explant	Average mean shoot length (cm)
0	43	47	+*	44[c]**	1.45 ± 0.49	0.55
0. 1	37	65	+	59[a,b,c]	2.00 ± 1.04	0.80
0.5	33	61	+	79[a,b,c]	2.26 ± 1.09	0.55
1.0	38	87	++	68[a,b,c]	2.73 ± 1.12	1.10
2.0	38	84	++	47[b,c]	1.83 ± 0.83	1.15
3.0	39	87	+++	90[a]	2.71 ± 1.11	1.05

*Relative amount of callus: (+) little, (++) good, (+++) profuse. ** Values in a column followed by the same superscript are not significantly different as determined by Chi-square test at 5% level of significance.

However, in most cases, only one of these shoots elongated significantly (Fig. 1C). As a result, each nodal explant ultimately yielded just one harvestable shoot. Alongside the development of shoots, light brown friable calli were formed. The quantity of these calli notably increased at the highest concentration of 2iP (3 mg/l), predominantly developing in the lower portion of explants. Nodal explants cultured at different zeatin levels formed

clustered shoot buds. In many cultures, only a single bud underwent differentiation to form a shoot (Table 4).

Table 4. Morphogenic response of *A. auriculiformis* nodal segments cultivated on N medium supplemented with zeatin. Data recorded after 65 days of inoculation. Experiment was conducted in April and repeated in May.

Zeatin (mg/l)	Number of explants	Explants forming callus (%)	Degree of callus formation	Explants forming shoots (%)	Average number of shoots per explant	Average mean shoot length (cm)
0	30	77	+*	87[a]**	1.84 ± 0.81	1.30
0.1	36	89	+	94[a]	1.76 ± 0.87	1.15
0.5	29	72	+	83[a]	1.41 ± 0.70	1.20
1.0	28	75	+	93[a]	1.34 ± 0.55	1.50
2.0	31	84	+	97[a]	1.86 ± 1.25	1.75
3.0	32	84	+	81[a]	1.61 ± 0.78	1.80

*Relative amount of callus: (**+**) little, (**++**) good, (**+++**) profuse. ** Values in a column followed by the same superscript are not significantly different as determined by Chi-square test at 5% level of significance.

The remaining buds located at the base of the shoot did not develop further and remained stunted (Fig. 1D), even after 65 days of incubation. In summary, while all four cytokinins tested supported the induction of multiple buds, their varying effects on subsequent differentiation and shoot development were quite pronounced. Different auxins adjuvant to the optimum BA level (1 mg/l) did not enhance the morphogenic response in terms of multiple shoot differentiation. In fact, auxins at higher levels, along with BA, reduced the vigour of shoots. Such antagonistic effects of auxin with cytokinin have also been previously noted in other leguminous species such as *Dalbergia sissoo* (Kumar et al. 1991) and *Bauhinia purpurea* (Kumar 1992). Efforts were undertaken to enhance the number of shoots generated per explant while simultaneously reducing the incubation period. Pursuing this goal, the inclusion of Ad.S. into N medium, along with BA supplementation, notably improved both the occurrence of responsive cultures and the number of shoots developing on each explant. The influence of Ad.S. on the induction of multiple shoots was evaluated using two distinct sets of experiments. The first approach involved using various concentrations of Ad.S. (0.1, 0.5, 1.0, and 1.5 mg/l) *ab inito* to the optimal level of BA (1 mg/l) and maintaining them for a period of 60 days without any subculture. The second approach entailed initially maintaining the explants on a BA containing medium for 30 days and subsequently transferring them to fresh N medium supplemented with the same concentration of BA, along with the above-mentioned levels of Ad.S., for additional 30 days. Ad.S. along with BA was more effective in the first set, *i.e.* when added to the medium in the earlier phase of incubation (Table 5).

Table 5. The morphogenic response of *A. auriculiformis* nodal segments on Ad. S. added to N medium containing 1mg/l BA. Data recorded after 60 days of culture. Experiment was conducted in May and repeated in June.

BA (mg/l)	Ad.S. (mg/l)	Number of explants	Explants forming callus (%)	Degree of callus formation	Explants forming shoots (%)	Average number of shoots per explant	Average shoot length (cm)
0.0	0.0	29	86	+*	76[a]***	2,04 ± 0.97	0.80
1.0	1.0	37	81	+	78[a]	4.03 ± 1.65	1.05
1.0	0.1	26	88	+	88[a]	5.56 ± 2.49	1.15
1.0	1.5	34	91	+	97[a]	4.06 ± 1.55	1.20
1.0	1.0	24	100	+	87[a]	4.28 ± 1.35	1.55
1.0	1.5	38	92	+	84[a]	3.64 ± 1.47	1.70
1.0	0.1**	29	83	+	79[a]	5.00 ± 2.39	1.10
1.0	0.5**	26	77	+	73[a]	4.10 ± 1.77	1.60
1.0	1.0**	33	70	+	79[a]	4.46 ± 1.78	1.20
1.0	1.5**	34	76	+	94[a]	3.37 ±1.79	1.45

*Relative amount of callus: (**+**) little, (**++**) good, (**+++**) profuse. **Sub cultured to the given combinations of Ad. S. and BA after 30 days of initial incubation on BA alone containing medium. *** Values in a column followed by the same superscript are not significantly different as determined by Chi-square test at 5% level of significance.

The average shoot number per explant was more than five on 0.1 mg/l Ad.S. In fact, in some cultures the *de novo* formed shoots were so many that it was difficult to count their numbers (Fig. 1E). The growth regulatory properties of Ad.S. had been demonstrated way back in 1948 by Skoog & Tsui and later by Miller and Skoog in the year 1953 in *Nicotiana tabacum*. Synergistic effect of Ad.S. and cytokinin in the present study also reinforces conclusions drawn on *Acacia nilotica* (Singh et al. 1993) and *Azadirachta indica* (Biswas and Gupta 1995).

Rhizogenesis: Both, the MS and N media (half and full-strengths) either alone or supplemented with IAA or NOA as well as Knop's medium failed to induce any roots. Only callus was formed at the base of shoots in IAA containing medium. NAA at 1 mg/l level in half-strength N medium supported 1-3.5 cm long roots in axenically raised shoots, *via* callus within 10-15 days of subculture if a pulse treatment of 3 mg/l of NAA was given for 30 min. Though IBA at 1 mg/l concentration in N medium also supported rhizogenesis but root formation was invariably preceded by the development of substantial amount of compact, white callus at the basal end. Whereas, in NAA containing medium, callus was meagre which was completely suppressed by adding AC at low concentration (0.01%; Fig. 2A). At the same time, it increased the frequency of rhizogenesis from 33% to 50%. The regenerated plantlets (Fig. 2B) showed good growth under *in vitro* conditions and they were gradually hardened for their subsequent transfer to field. In conclusion, the present study demonstrates the capacity of *A. auriculiformis* to be clonally propagated under *in vitro* conditions through nodal explants excised from old

trees, with an aim of eventually extending the much desired forest cover which is depleting at unprecedented rate.

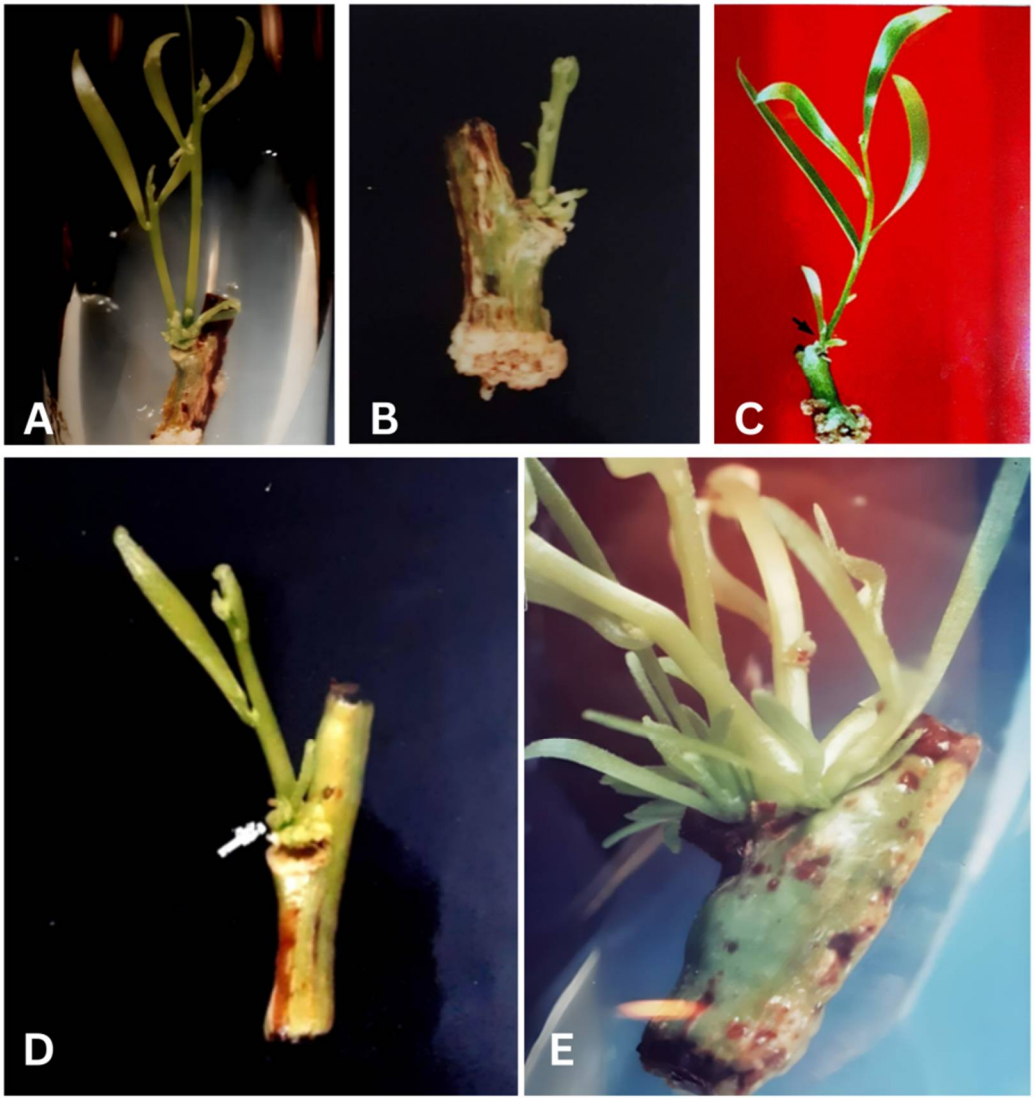

Fig. 1(A-E). Morphogenic responses of nodal explants excised from 30-year-old *Acacia auriculiformis* tree and cultured on Nitsch's medium, under 16 hr photoperiod. A. Healthy multiple shoots differentiated on 1 mg/l BA, after 65 days of inoculation. x 2.5, B. Under-developed shoots with diminutive phyllodes on 0.1 mg/l Kn, after 65 d. of culture. x 3, C. A long shoot with well-developed phyllodes and a stunted shoot at its base (arrow) as well as callus at the basal end of the explant, implanted on 3 mg/l 2iP, after 65 days of culture. X 1.5, D. A single shoot with a cluster of under-developed buds (arrow) at its base on 2 mg/l zeatin, after 65 days of inoculation. x 3.5, E. Profusely developed multiple shoots on a nodal explant, cultured on BA and 0.1 mg/l Ad.S., after 60 days of culture. x 5.

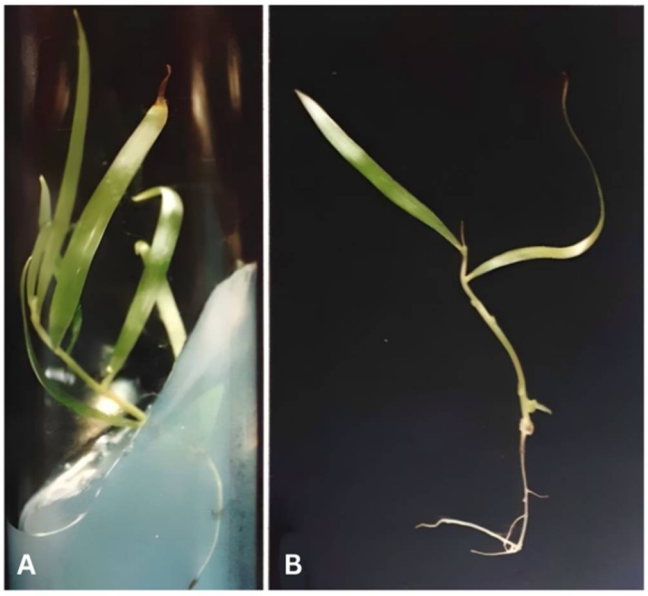

Fig. 2(A-B). Rhizogenesis in axenically raised shoots of *Acacia auriculiformis*. A. Direct root formation with prominent root cap on 0.01% AC supplemented to N (half-strength) along with 1 mg/l NAA after 15 days of culture. x 1.6, B. Axenically raised plantlet with branched root system on 1 mg/l NAA adjuvanted to N medium (half-strength), after 30 days of culture. x 1.

In conclusion, this research presents a novel and highly effective approach to optimizing shoot multiplication and rooting efficiency in 30-year-old *A. auriculiformis* trees, leading to robust plantlet production. The utilization of Nitsch's medium supplemented with 1 mg/l N6-benzyladenine (BA) induced the formation of multiple shoots in nodal explants, a significant breakthrough in the propagation of mature *A. auriculiformis* trees. However, the true innovation lies in the addition of 0.1 mg/l adenine sulfate to the N medium containing BA, which remarkably enhanced the caulogenic response. This resulted in a substantial increase in the number of shoots developing on each node, elevating the yield from three to an impressive fifteen shoots per node. Moreover, the successful axenic rooting of the shoots on N medium (half strength) with 1 mg/l naphthalene acetic acid further exemplifies the efficiency of this methodology in obtaining healthy plantlets. The incorporation of activated charcoal (0.01%) in the medium significantly contributed to suppressing callus formation at the root-shoot junction, ensuring the development of vigorous and disease-free plantlets. What sets this research apart is its focus on the propagation of 30-year-old *A. auriculiformis* trees, which presents unique challenges due to the age and maturity of the plant material. The innovative use of adenine sulfate supplementation to enhance shoot multiplication is a standout feature, offering a practical and efficient method for increasing the yield of shoots. This research not only contributes to the field of plant propagation but also holds

promise for the conservation and commercial cultivation of *Acacia auriculiformis*, given the robust plantlet production achieved. The findings have the potential to revolutionize the propagation techniques for mature trees, thus making a significant impact on afforestation and agroforestry efforts.

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