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Hormonal Regulation Impact on Regeneration of *Acacia nilotica* L. a Nitrogen Fixing Tree

K. Dhabhai and A. Batra

Department of Botany, University of Rajasthan, Jaipur, Rajasthan 302055, India

Abstract: An efficient regeneration protocol was developed for the establishment of *Acacia nilotica* L., a nitrogen fixing, multipurpose leguminous tree, through indirect organogenesis. Present investigation was the first attempt to obtained indirect organogenesis in *Acacia nilotica* L. through cotyledonary node explant of *in vitro* grown plants. Cotyledonary node (1.0 cm) explant excised from 20 day old in vitro seedling cultured on MS medium supplemented with various concentrations of 2, 4- D alone and in combination with BAP for callus induction. However, hormonal regulation and their concentrations played crucial role in obtaining the callus. Under the optimized conditions, maximum and rapid growth of green callus was obtained after the 25 days of inoculation on MS medium with 2, 4- D (2.0 mg/l) alone or in combination with of 2, 4- D (0.40 mg/l) and BAP (0.20 mg/l) in combination. Combination of auxin and cytokinin proved to be more effective for callus induction. Shoot induction was also obtained on the same medium after subculturing twice at the time interval of 21 days. The highest number and their elongation of adventitious shoots achieved when activated charcoal (200 mg/l) added on MS medium fortified with 2, 4- D (0.40 mg/l) and BAP (0.2 mg/l). However, the elongated adventitious shoots were rooted on half strength MS medium with IBA (0.5 mg/l) after 20 days. Rooted plantlets were hardened and successfully established in soil with 75% survival rate.

Key words: Indirect regeneration · Auxin · Cytokinin
Abbreviations: 2,4-dichlorophenoxy acetic acid (2,4-D), 6-Benzylaminopurine (BAP), Murashige and Skoog medium (MS)

INTRODUCTION

Acacia nilotica (L.), a nitrogen fixing tree commonly known as Babool or Kikar, belongs to the family Leguminosae. It is found in India, Australia and Africa. This plant has been used as a source of pulpwood, timber, fodder and gum. It has been proved as effective medicine in treatment of malaria, sore throat (aerial part) and toothache (bark) [1, 2]. The methanolic extracts of *Acacia nilotica* pods have been claimed effective against HIV-PR [3,4]. The fresh parts of this plant have been reported to be most active against Hepatitis C virus [5]. This plant also shows antioxidant activity and antimicrobial activity [6,7]. It acts as a biological agent for herbivores [8].

The leguminous trees are one of the most significant components of forest vegetation due to their economic and ecological importance. In general, the woody plants are difficult to regenerate under *In vitro* conditions but some success was achieved in a few leguminous tree species through direct regeneration in Acacia species like *A. chundra* (Roxb.).DC [9] and *A. Senegal* [10] but least success was reported via indirect regeneration in Acacia species as in *A. crassicarpa* [11]. *In vitro* regeneration protocols of *Acacia nilotica* L. through indirect organogenesis have been standardized earlier from endosperm repeatedly, culture [12]. Hence, present investigation was the first attempt to obtained indirect organogenesis in *Acacia nilotica* L. through cotyledonary nodes.

The regeneration system here described for *Acacia nilotica* L. is innovative and reproducible.

MATERIALS AND MATERIALS

Establishment of Aseptic Seedlings: Mature seeds of *Acacia nilotica* L. were collected from Sirsi (Haatoj) District, Jaipur, Rajasthan. Prior to surface sterilization, seeds were treated in boiled water at 60°C for about 30 mins.and then soaked in distilled water for about 24 hrs. Then they were kept under running tap water for about 10-15 mins. followed by Washing with 1 % (v/v) Rankleen (Ranklem-India) for 2 mins. and rinsed with double

Corresponding Author: K. Dhabhai, Department of Botany, University of Rajasthan, Jaipur, Rajasthan 302055, India.
E-mail: kshipramicrobiology@gmail.com, amlabatra@gmail.com.

distilled water for three times. Prior to inoculation, sterilized seeds were again sterilized with 0.1% (w/v) aqueous $HgCl_2$ for about 2 mins. followed by 2-3 rinsing with double distilled water in Laminar Air flow cabinet. These sterilized seeds were inoculated on half and full strength MS salts medium in cultured bottles, filter paper bridges in culture tubes, also on filter paper disk and non-absorbent cotton in the Petri plates. After 7-10 days of inoculation, seeds germinated and gave rise seedlings (Table 1, Figure A). These *in vitro* seedlings were used as source of explants.

Culture Media & Conditions for in Vitro Grow Plant via Indirect Organogenesis: Cotyledonary nodes (1.0 cm) were procured from the seedlings, which doesn't not required sterilization and inoculated on the MS medium congealed with various concentrations of cytokinin and auxins for the morphogenic responses.

However, MS medium was tried as control for a series of experiments in addition to hormones. The Murashige and Skoog (MS) medium was prepared by adding 3% sucrose as a carbon source and 0.8% (w/v) agar as a solidifying agent. In vitro cotyledonary node (1.0 cm) of 20 day old seedlings inoculated as explants for callus induction on MS medium supplemented with a series of 2,4-D (0.5-4.0mg/l) alone or in combinations with 2,4-D (0.2-2.0 /l) with BAP (0.1-1.0 mg/l). Adventitious shoots were raised from callus after subculturing twice on combination medium 2,4-D and BAP in combination. Activated charcoal at the range (100-1000 mg/l) added as an antioxidant for triggering the number and elongation of shoots.

In vitro regenerated shoots measuring about 3.5-4.0 cm grown in multiplication medium were excised and cultured on half-strength MS basal medium as control and supplemented with IBA (0.1-0.7 mg/l) and NAA (0.2-1.0 mg/l) alone for root emergence. The pH of medium was adjusted to 5.8 ± 0.02 before autoclaving at 121°C for 15 minutes at 15 lb/in^2. 20 ml of molten agar medium was poured into a culture bottle and plugged with nonabsorbent cotton. All cultures were incubated in 16 h /8 h photoperiod under light intensity of 50 µE/ m^2/s provided by cool, white and fluorescent light (40 V) (Philips, India) at 25 ± 2°C with 55% relative humidity. Each treatment performed using eight replicates and the experiment was repeated at least thrice. The cultures were examined every day and the morphological changes were recorded on the basis of visual observations. After then in vitro grown plants were transfer for hardening and acclimatization.

Acclimatization: The plantlets were taken out from culture tube without damaging the delicate root system and rinsed with distilled water to remove adhering agar and then transferred to polycups containing vermicompost and autoclaved soil (1:3). Polycups were covered with inverted glass beakers to maintain high humidity and kept in culture chamber. After four weeks, acclimatized plants were transferred in a greenhouse under normal day length conditions for gradually exposed from artificial environmental conditions to natural acclimatization.

Data Analysis: The data were collected after two weeks for shoot regeneration and two weeks for rooting experiments. There were eight replicates and one explant was cultured per bottle. The experiments were repeated thrice. The effect of different growth regulators were statistically analyzed and means were separated at $p<0.05$ level of significance. Standard errors (SE) of the difference between treatments are presented.

RESULT AND DISCUSSION

During the present set of experiment, no results were achieved on control MS medium regarding callus induction, proliferation of shoots, multiplication of shoots and *In vitro* -root formation. Various tests were performed to germinate seeds *In vitro* conditions (Table 1). Out of all the tests performed, filter paper bridges proved to be most effective with 90% response for *in vitro* seed germination. The results obtained on seed germination in *Macrotyloma uniflorum* also supported the present results [13]. These *In vitro* germinated seedlings were the regular source of cotyledonary node explant in further experiments. The present investigation was carried out to explore the morphogenic potential of *A. nilotica* by using different combinations of growth regulators. Hormonal regulation and their concentrations played crucial role in obtaining the callus. Callus was initiated when *in vitro* cotyledonary node (1.0 cm) explants cultured on MS medium supplemented with 2, 4- D (0.2-2.0 mg/l) and

Table 1: In vitro seed germination methods:

S.Number	Methods	Days taken	Percentage (%)
1	Filter paper bridge method	5	90
2	Filter paper disk method	7	75
3	Cotton disk method	10	68
4	Half strength MS medium	15	55
5	MS medium	25	20

Table 2: Effect of Plant Growth Regulators on callus induction from cotyledonary node after 4 weeks

Media combinations (mg/l)		% Response (Mean ± SE)*	Nature of Callus**
2, 4- D	2, 4- D+ BAP		
0.5		55.4 ± 0.7	C, P, Lw
1.0		60.3 ± 0.9	C, P, Md
1.5		64.1 ± 0.6	C, G, Md
2.0		70.2 ± 0.4	C, G, Lg
2.5		51.2 ± 0.8	C, P, Lw
3.0		49.7 ± 0.8	C, P, Lw
3.5		45.2 ± 0.5	C, P, Lw
4.0		40.1 ± 0.2	C, P, Lw
4.5		38.2 ± 0.1	C, P, Lw
5.0		32.5 ± 0.9	C, P, Lw
	0.2+0.1	69.7 ± 0.5	F, G, Lg
	0.4+0.2	75.7 ± 0.3	F, G, Lg
	0.6+0.3	67.2 ± 0.6	F, G, Lg
	0.8+0.4	63.9 ± 0.2	F, P, Md
	1.0+0.5	53.6 ± 0.4	F, P, Lw
	1.2+0.6	53.5 ± 0.5	F, P, Lw
	1.4+0.7	51.2 ± 0.5	F, P, Lw
	1.6+0.8	45.1 ± 0.3	F, P, Lw
	1.8+0.9	36.3 ± 0.1	F, P, Lw
	2.0+1.0	30.0 ± 0.7	F, P, Lw

*Values are 95% confidence limits for mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

** C: compact, F: friable, G: green, Lg: large, Lw: low, p: pale

BAP (0.1-1.0 mg/l) in combination. 2, 4- D (0.5-5 mg/l) alone only started the callus initiation but when this callus subcultured on MS medium along with 2, 4-D and BAP produced stock callus. Nature of callus varied with hormones and their concentrations. This was proved by response of obtained callus, as compact callus observed on 2, 4- D alone or friable callus on combination of 2, 4- D with BAP on MS medium. These results were coincides with *Vigna aconifolia* [14]. Combination of auxin as 2, 4- D and cytokinin like BAP proved to be more effective for callus induction.

This combination medium in the series of 2, 4- D (0.2 - 2.0 mg/l) with BAP (0.1- 1.0 mg/l) gave rise to shoot proliferation after subculturing twice at the time interval of 21 days. Hormones have been considered to act by reacting with a specific target site of the known plant hormones. The cytokinins, because of their role in experimentally induced cell division and differentiation, serve as a probe of hormonal involvement in differentiation [15]. Activated charcoal improves cell growth and development. Hence, to obtained highest number and their elongation, activated charcoal added as an antioxidant in the range (100-1000 mg/l). The promontory effects of activated charcoal on morphogenesis is mainly due to its irreversible adsorption of inhibitory compounds in the culture medium with substantially decreasing the toxic metabolites, phenolic exudation and brown exudates accumulation [16]. Maximum number of shoots (5.80 ± 0.4) obtained on MS medium augmented with 2, 4- D (0.4 mg/l), BAP (0.2 mg/l) and AC (200 mg/l). Similar results were reported

Table 3: Effect of Activated Charcoal with auxin and cytokinin on adventitious shoot induction from callus of *A. nilotica* L. after 2 weeks of twice subcultured

Media combinations (mg/l)			% Response	Mean Average Number of multiple	Average length of shoots
2,4- D	BAP	AC	(Mean ± SE)*	shoots (Mean ± SE)*	(Mean ± SE)*
0.2	0.1	100	30.2 ± 0.1	3.75 ± 0.9	2.6 ± 0.2
0.4	0.2	200	78.6 ± 1.2	5.80 ± 0.4	4.0 ± 0.6
0.6	0.3	300	69.2 ± 0.3	3.10 ± 0.2	3.5 ± 0.3
0.8	0.4	400	64.9 ± 0.8	2.99 ± 0.3	3.3 ± 0.5
1.0	0.5	500	53.6 ± 0.5	2.06 ± 0.1	2.9 ± 0.7
1.2	0.6	600	45.2 ± 0.2	1.98 ± 0.7	2.7 ± 0.6
1.4	0.7	700	40.1 ± 0.4	1.75 ± 0.9	2.5 ± 0.4
1.6	0.8	800	38.8 ± 0.6	1.55 ± 0.5	2.3 ± 0.2
1.8	0.9	900	35.6 ± 0.3	1.43 ± 0.3	2.1 ± 0.1
2.0	1.0	1000	30.1 ± 0.1	1.25 ± 0.1	1.5 ± 0.8

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 5% or 0.05 probability level

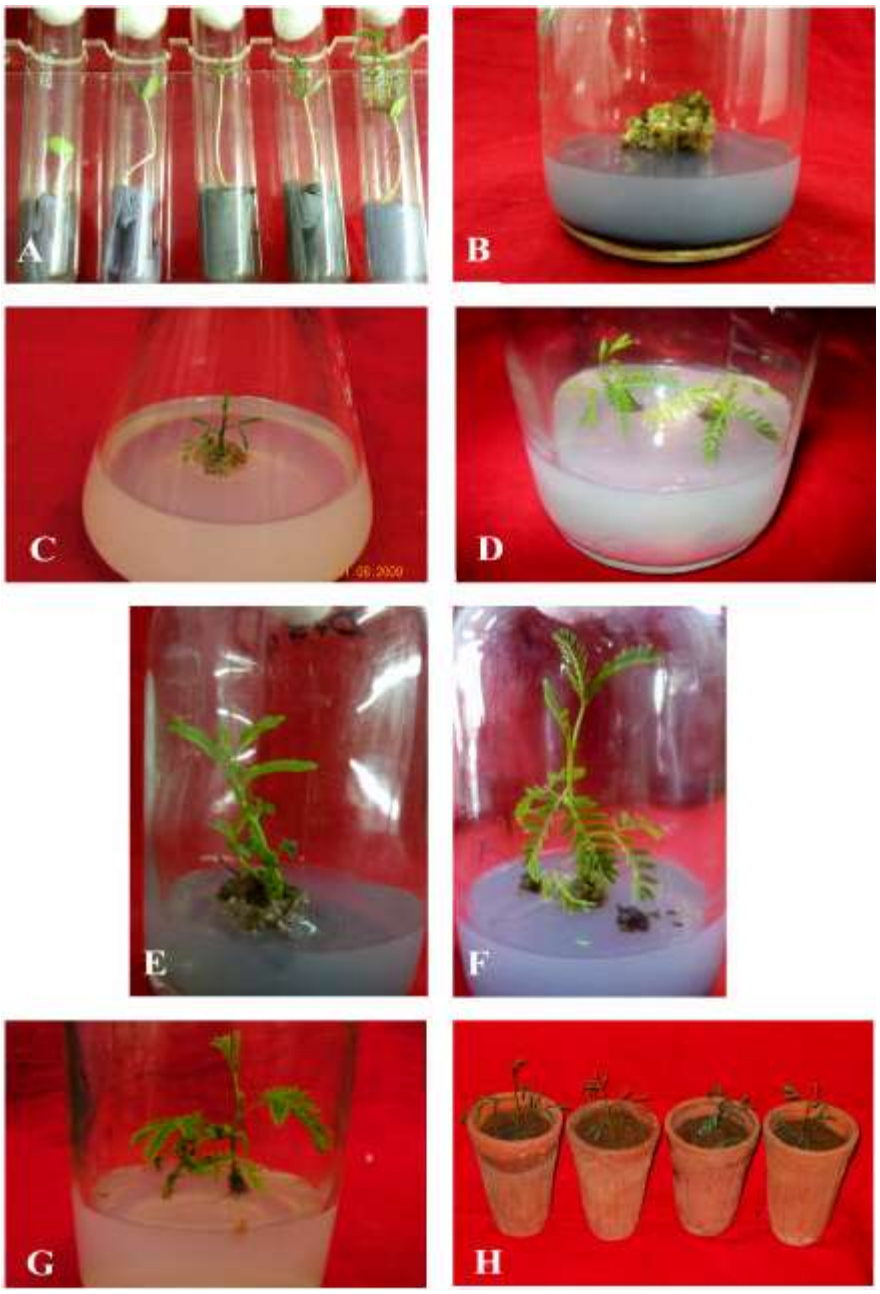

Fig. A: *In vitro* seed germination on filter paper bridges
Fig. B: Induction of callus from cotyledonary node of *Acacia nilotica* on MS medium supplemented with 2, 4- D (2 mg/l)
Fig. C: Shoot proliferation of *Acacia nilotica* on MS medium in combination of 2, 4- D (0.4 mg/l) and BAP (0.2 mg/l) after twice subculturing of callus
Fig. D: Proliferation of shoots of *Acacia nilotica* on MS medium in combination of 2, 4- D (0.4 mg/l) and BAP (0.2 mg/l) after thrice subcultring of callus
Fig. E: Induction of multiple shoots from callus of *Acacia nilotica* on MS medium with combinations of 2,4-D (0.4 mg/l), BAP (0.4 mg/l) and Activated charcoal (200 mg//l).
Fig. F: Induction of rooting from elongated shoots of *Acacia nilotica* on ½ MS medium augmented with IBA (0.5 mg/l)
Fig. G: Hardened plantlet of *Acacia nilotica* growing in a garden soil after 25 days of transfer

Table 4: Effect of Auxins on rooting of *Acacia nilotica* L. after two weeks of culture

Plant Growth Regulators (mg/l)			
NAA	IBA	% response	% of rooting (Mean ± SE)*
0	0	0	0
0.2		10	1.4 ± 0.6
0.4		50	2.5 ± 0.6
0.6		80	3.5 ± 0.7
0.8		60	3.2 ± 0.8
1.0		20	1.6 ± 0.4
	0.1	20	1.6 ± 0.4
	0.3	40	2.4 ± 0.6
	0.5	90	3.6 ± 0.6
	0.7	30	2.1 ± 0.4
	0.9	60	3.2 ± 0.8

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 5% or 0.05 probability level

in *Phaseolus vulgaris* L [17]. In contrary to the present results, in *Populus euphratica* olive [18] and in *Acacia crassicarpa* [11], TDZ and NAA proved to the be best for indirect organogenesis instead of 2, 4- D and BAP.

Elongated shoots derived from callus were separated and cultured on half strength MS basal medium supplemented with IBA (0.5 mg/l) and NAA (0.6 mg/l) alone for induction of rooting. Out of the auxins tested, IBA induced rooting at the basal end of shoots (Table 2). The optimum concentration was 0.5 mg/l of IBA and it resulted in 80% of root formation within 13-15 days of culture (Figure D). At higher concentrations (1.0–1.5 mg/l) of IBA, the percentage of rooting was reduced and callus formation was obtained at the basal cut end. Similar results were observed in *A. mangium, Withania somnifera, A. Senegal* and *Prosopis ceneria* for root induction [19,20,21] respectively. In contrary to the present results, MS medium alongwith IAA (2 mg/l & 3 mg/l) respectively in *A. nilotica, A. catechu, Ormacarpum sennoides* and *Withania somnifera* for root induction [22-25] respectively.

Now, efforts are being made to gradually transfer plant to the field. After 35-45 days of culturing the shoots on rooting medium, the plantlets were removed from the cultured tube and acclimatization was done by the method described in "Materials and Methods" (Figure E).

CONCLUSION

Due to overexploitation and uncontrolled utilization of *Acacia nilotica* for commercial reasons, this plant needs *In vitro* regeneration. Hence, this paper presents quick, reliable and reproducible for regeneration of *A. nilotica* through hormonal regulation without any seasonal influences. However, few reports are also available on regeneration of this plant species, but they are not much efficient and taking more time as compared to the present protocol.

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