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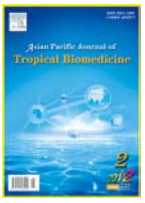

Document heading

Rapid *in vitro* multiplication of the ethnomedicinal shrub, *Acacia caesia* (L.) Willd. (Mimosaceae) from leaf explants

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ABSTRACT

Objective: To develop an efficient protocol for *in vitro* multiplication of the ethnomedicinal shrub *Acacia caesia* (*A. caesia*) L. Willd., **Methods:** Leaf explants were inoculated on MS medium supplemented with TDZ and NAA for callus induction. Subculturing experiments were conducted by using leaf derived calli for shoot proliferation on MS medium fortified with various growth regulators like IBA, TDZ, BAP and GA3. The regenerated shoots were rooted in half strength MS medium supplemented with various concentrations of IBA, IAA and Kn. After roots were developed, the plantlets were transplanted to pots filled with garden soil, sand and vermicompost and kept in growth chamber with 70%-80% humidity under16h photoperiod. After acclimatization, the plantlets were transferred to the garden and survival percentage was calculated. Data were statistically analyzed and means were compared using Duncan's Multiple Range Test ($P<0.05$). **Results:** An *in vitro* multiplication protocol was developed for the locally demanded medicinal plant species, *A. caesia* by using leaf explant. The study revealed that the callus formation was effective in MS medium containing TDZ and NAA at 1.5 and 0.3 mg/L respectively. Shoot induction was most successful in MS medium supplemented with combination of the auxin, IBA and cytokinin, TDZ at 2.0 and 0.5 mg/L respectively. A single leaf explant was capable of producing 12 shoots/callus after 30 days of culture. The other supplementation in MS medium with IBA and Kn at 2.0 and 0.4 mg/L respectively produced higher rooting frequency, roots/shoot and root length. The survivability rate of leaf callus derived plantlets was significantly higher (84%) in the hardening medium composed by garden soil, sand and vermicompost (1:1:1) by volume. **Conclusions:** A significant progress has been made in the *in vitro* regeneration system of this medicinally important plant species, *A. caesia*.

1. Introduction

Mass propagation of plant species through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently it has been much progress in this technology for some medicinal plants. Clonal propagation of high - value forest trees through organogenesis has the potential to rapidly capture the benefits of breeding or genetic engineering programs and to improve the quality and uniformity of nursery stock. Plant tissue culture is well known biotechnological tool for the rapid propagation of medicinal plants for the purpose of commercialization and conservation[1].

Acacia caesia (*A. caesia*) (L.) Willd. is an armed woody straggling shrub of medicinal importance belongs to the family, Mimosaceae, and it is widely distributed in the foot hills of the Western Ghats around the altitude of 500 ms above msl. For its medicinal properties, the traditional healers of western districts of Tamil Nadu (Tirupur, Coimbatore, Erode and Dindugal) prescribed well for various ailments. This species is a number of the speculated medicinal properties have been validated by scientific research. These include leaves were used as vegetable and in the treatment of asthma, skin diseases[2,3], menstrual disorder[4-7], scabies[8]. Due to these values, it is exploited severely by the local public in western districts of Tamilnadu and it becomes a rare sighted species in the natural habitats of Western Ghats[9]. Hence to meet the demand, *A. caesia* requires mass propagation through *in*

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vitro regeneration attempts.

2. Materials and methods

For surface sterilization, healthy and immature leaf segments were collected from three months old individuals of *A. caesia* grown under greenhouse condition and used as explants. These segments were washed under running tap water followed by treatment with a surfactant, tween 20 (5% w/v) for 5 min. After repeated washes in double distilled water, to eliminate the fungal contamination, the explants were treated with carbendazim (50% w/v) fungicide (10%) for 15 min and rinsed with double distilled water 2 or 3 times. To eliminate bacterial contamination, the explants were also treated with 5% antibiotics (Ampicillin and Rifampicin) for 30 min followed by three rinses in sterile double distilled water. Furthermore, surface sterilization was carried out by dipping the explants in 0.1% $HgCl_2$ for 3 min followed by 3-4 rinses in sterilized double distilled water. Explants were inoculated on MS[10] medium supplemented with thidiazuron (TDZ: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) and were tested with α-naphthalene acetic acid (NAA: 0.3 mg/L) or indole 3-acetic acid (IAA: 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L) or 6-benzyl amino purine (BAP: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) for callus induction.

To initiate the effective shoot formation from leaf callus derived explants, subculturing experiments were carried out by using MS medium with folowing growth regulators. Indole 3-butric acid (IBA: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) along with TDZ (0.5 mg/L) for shoot induction were used for this purpose.

The regenerated shoots (2 to 3 cm) were rooted on half strength MS medium supplemented with various concentrations and combinations of IBA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) or IAA (0.3 mg/L) or Kn (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/L). After the development of roots, the plantlets were transplanted to pots filled with garden soil, sand and vermicompost (1:1:1 by volume) and kept in growth chamber with 70%-80% humidity under 16 h photoperiod. After acclimatization, the plantlets were transferred to the garden and survivability percentage was calculated.

Each experiment was done at least twice using triplicate. Data were statistically analyzed and means were compared using Duncan's Multiple Test ($P<0.05$).

3. Results

In this present study, growth regulators in different concentrations and combinations are used to determine the optimal culture conditions to regenerate the leaf explants of *A. caesia in vitro*. Table 1 showed that the leaf explant responsed well (83%) for callus formation on MS medium

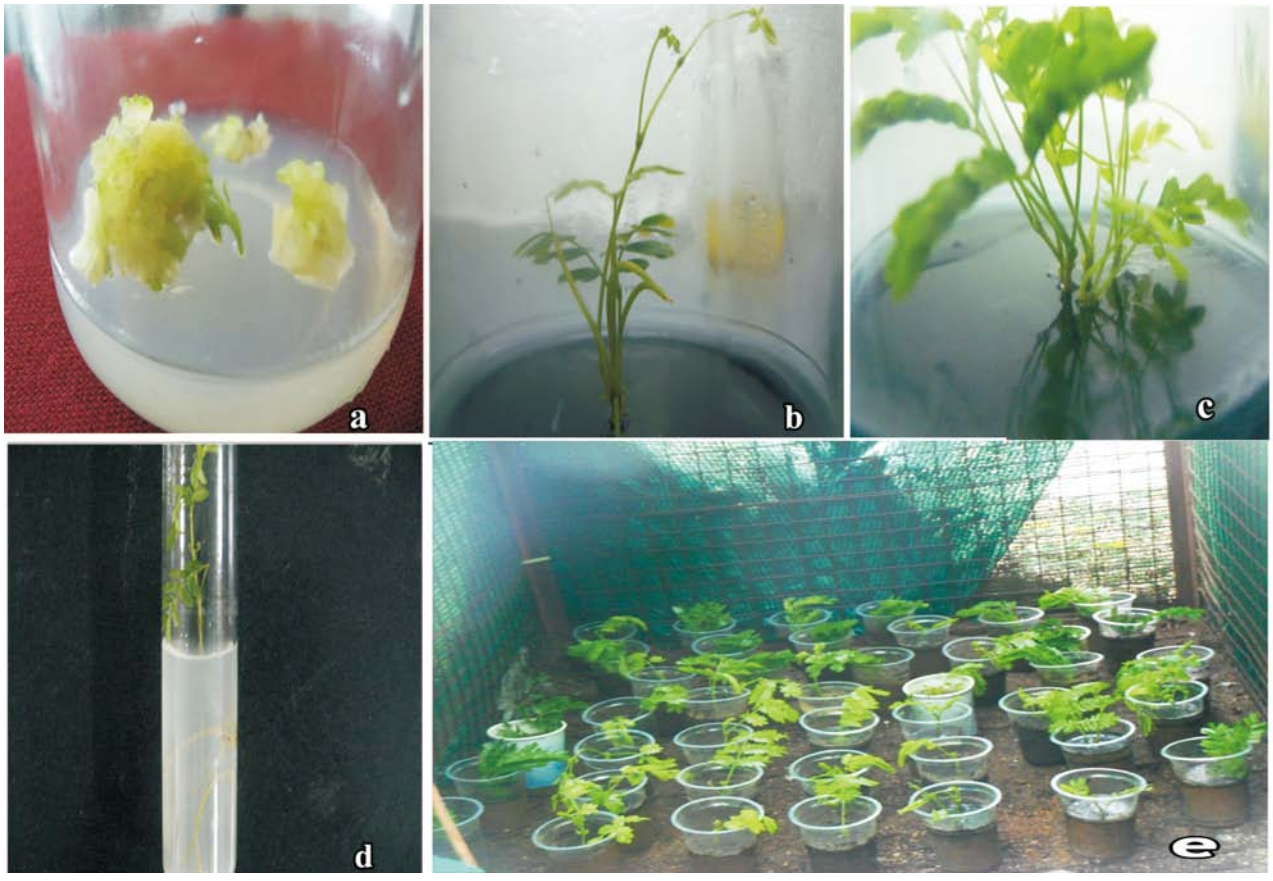

Figure 1. *In vitro* regeneration through leaf explant of *A. caesia*.
a: Effective callusing in MS medium supplemented with TDZ and NAA at 1.5 and 0.3 mg/L respectively. b: Successful shooting by subculturing of leaf derived callus in the MS medium with IBA and TDZ at 2.0 and 0.5 mg/L respectively. c: Multiple shoot formation by subculturing of *in vitro* derived shoots in the MS medium with IBA and TDZ at 2.0 and 0.5 mg/L respectively. d: High amount of rooting during the subculturing of shoots in the MS medium fortified with IBA and Kn at 2.0 and 0.4 mg/L respectively. e: Under hardening in the mist chamber.

Table 1.
Effect of growth regulators on callus induction in *A. caesia*.

Conc. of growth regulators (mg/L)				Leaf explant	
NAA	TDZ	BAP	IAA	Days required	Callus formation (%)
–	0.5	–	–	23	59.31 ± 0.82 g
–	1.0	–	–	23	68.22 ± 1.63 h
–	1.5	–	–	30	83.23 ± 0.82 j
0.3	2.0	–	–	19	9.44 ± 1.63 l
–	2.5	–	–	6	0.00 ± 1.63 i
–	3.0	–	–	28	80.18 ± 0.82 i
–	–	0.5	–	18	25.21 ± 1.63 b
–	–	1.0	–	20	41.38 ± 1.63 d
–	–	1.5	–	22	58.45 ± 1.63 g
0.5	–	2.0	–	24	69.64 ± 0.82 h
–	–	2.5	–	28	78.32 ± 1.63 i
–	–	3.0	–	27	78.17 ± 1.63 i
–	0.3	–	0.2	16	15.89 ± 0.82 a
–	0.6	–	0.4	17	23.43 ± 0.82 b
–	0.9	–	0.6	18	41.00 ± 0.82 d
–	1.2	–	0.8	20	46.00 ± 1.63 e
–	1.5	–	1.0	19	47.00 ± 1.63 e

Means in columns followed by different letter are significant to each other at 5% level according to DMRT.

Table 2.
Effect of growth regulators on shoot initiation, shoot number and shoot length in *A. caesia*.

Conc. of growth regulators (mg/L)		Leaf explant		
TDZ	IBA	Culture response (%)	Number of shoots/callus	Shoot length (cm)
–	0.5	58.48 ± 1.63 h	6.65 ± 0.82 cde	3.80 ± 1.63 abc
–	1.0	63.00 ± 0.82 i	8.49 ± 1.63 efg	4.20 ± 0.82 abc
–	1.5	78.43 ± 1.63 j	7.38 ± 2.45 def	4.90 ± 1.63 bc
0.5	2.0	85.00 ± 0.82 k	12.61 ± 1.63 i	5.60 ± 0.82 c
–	2.5	63.67 ± 1.63 i	11.47 ± 1.63 hi	5.30 ± 1.63 bc
–	3.0	58.46 ± 1.63 h	10.98 ± 0.82 ghi	4.70 ± 0.82 abc

Means in columns followed by different letter are significant to each other at 5% level according to DMRT.

Table 3.
Effect of growth regulators on rooting percentage, root number and root length in *A. caesia*.

Conc. of growth regulators (mg/L)			Leaf explant		
		Kn	Shoots rooted (%)	Number of roots/shoot	Root length (cm)
IBA	0.5	–	40.36 ± 0.82 b	4.35 ± 0.82 abc	3.50±0.41 a–d
	1.0	–	48.79 ± 1.63 d	4.76 ± 0.41 abc	2.80±0.65 ab
	1.5	–	52.59 ± 0.82 e	5.48 ± 1.63 bcd	3.00±0.82 abc
	2.0	–	57.43 ± 0.41 g	4.25 ± 0.82 abc	4.80±0.65 c–f
	2.5	–	60.90 ± 1.63 h	6.46 ± 0.82 cde	5.40±0.33 ef
	3.0	–	48.00 ± 0.82 d	5.56 ± 1.63 bcd	4.00±0.82 b–e
	0.5	0.1	44.75 ± 0.65 c	4.26 ± 0.41 abc	3.00±0.82 abc
	1.0	0.2	53.47 ± 1.63 e	5.38 ± 0.82 bcd	3.00±1.63 abc
	1.5	0.3	65.38 ± 0.82 i	6.76 ± 0.82 cde	2.00±0.82 a
	2.0	0.4	72.69 ± 1.63 j	8.37 ± 1.63 e	6.00±0.82 f
	2.5	0.5	67.46 ± 0.82 i	7.65 ± 0.41 de	5.00±0.41 def
	3.0	0.6	56.57 ± 0.41 fg	5.98 ± 0.82 a	4.00±0.82 b–e
IAA	0.3	–	35.86 ± 1.63 a	2.43 ± 1.63 ab	1.50±1.63 abc
	0.3	–	41.45 ± 0.82 b	3.23 ± 0.82 ab	4.20±0.16 b–f
	0.3	–	48.34 ± 1.63 d	4.49 ± 1.63 abc	3.10±0.82 abc
	0.3	–	49.64 ± 0.82 d	5.75 ± 0.41 bcd	3.40±0.33 a–d
	0.3	–	52.28 ± 1.63 e	6.00 ± 1.63 cde	2.50±0.41 ab
	0.3	–	54.17± 0.82 ef	4.37 ± 0.82 abc	3.20±0.16 a–d

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT.

Table 4.
Effect of different composition of hardening medium on survivability of *A. caesia*.

Hardening medium composition (V/V)	No. of plantlets under hardening	No. of plantlets survived	Survivability (%)
Red soil + sand (1:1)	50	22	44.00 ± 1.63a
Garden soil + sand + vermicompost (1:1:1)	50	42	84.00 ± 0.82e
Decomposed coir waste + perlite + compost (1:1:1)	50	36	72.00 ± 0.41d
Vermicompost + soil (1:1)	50	30	60.00 ± 1.22c
Red soil + sand + vermicompost (1:1:1)	50	28	56.00 ± 0.82b

Means in columns followed by different letter are significant to each other at 5% level according to DMRT.

supplemented with TDZ and NAA at 1.5 and 0.3 mg/L respectively after 30 d of inoculation (Figure 1a). The leaf derived callus produced effective shoot formation (85%) (Figure 1b) in the MS medium containing IBA and TDZ at 2.0 and 0.5 mg/L respectively (Table 2) and average of 12.61 shoots with 5.6 cm shoot length were developed (Figure 1c).

Regenerated shoots (3-4 cm long) were transferred to half strength MS medium fortified with auxin at different concentrations for root induction (Table 3). Leaf callus derived shoots were more pronounced (72%) for all rooting attributes in the MS medium with IBA and Kn at 2.0 and 0.4 mg/L respectively and average number of 8.37 roots with 8.37 cm root length were developed (Figure 1d). The percentage of root induction was less in other supplementations of IAA and IBA in the MS medium. The rooted shoots were transferred to hardening media to determine the survivability rate of leaf callus derived plantlets. It was significantly higher (84%) in the hardening medium composed by garden soil, sand vermicompost in the ratio of 1:1:1 by volume followed by the hardening medium consists of decomposed coir waste, perlite and compost (72%) in the ratio of 1:1:1 by volume. More than 70% of the plants were noted to be survived in the field (Figure 1e).

4. Discussion

The *in vitro* propagation of woody species has been proven to be a viable method for the production of explants, which is of great value for the conservation of native species and the recovery of degraded areas as well[11-13]. Tissue culture technology offers an alternative method of reproduction and the conservation of germplasm as well for the medicinally important plant resources[14-16]. However, the degree of response is depending upon the species and number of other extrinsic factors[17]. In this study, the study species, *A. caesia* required a longer period of 30 d for callus induction. It may be due to the nature of tissues, the degree of totipotency and composition of medium with respect to micronutrients and hormones[18]. Arumugam *et al*[19] also reported the requirement of longer period of more than 35 days for effective callus formation in many species of *Acacia* and pointed out that several factors including the culture environments and hormonal and non hormonal regulators act synergistically in determining the proper induction, proliferation of calli and regeneration into plants.

The effective callus formation has occurred in the MS medium containing the cytokinin and auxin combination (TDZ and NAA) for the study species, *A. caesia*. Senthilkumar and Paulsamy[20] reported that the level of NAA is most important factor for callus formation in many species.

The leaf derived callus of *A. caesia* was highly effective for shoot initiation while subcultured onto the MS medium containing IBA at higher amount (2 mg/L) and TDZ lesser amount (0.5 mg/L). Basalma *et al*[21] already reported that the combination of IBA and TDZ was most effective for shoot formation in the species like *Carthamus tinctorius* rather than the individual treatment of IBA. It indicates that the presence of TDZ in traces amount in the MS medium induces high frequency of shoot regeneration. Similar kinds of observation on the requirement of cytokinin (TDZ) for better shooting were reported in many species[22-26].

The root induction during the subculturing of *in vitro* leaf callus derived shoots was significantly higher in the MS medium augmented with high quantity of IBA and low quantity of Kn for the study species, *A. caesia*. Arumugam et al[27] have already reported the requirement of high quantity of auxin with low quantity of cytokinin for the root formation of *Aegle marmelos* during the subculturing of secondary explants the shoots. Karuppusamy and Pullaiah[28], Mahendran and Narmatha Bai[29], Abbas *et al*[30] and Loc and Kiet[31] have also reported the importance of auxins in the root formation during the subculturing of secondary explants. Johri and Mitra[32] explained that auxin and cytokinin act synergistically to regulate the process of cell division and hence the growth of root or shoot. Frank *et al*[33] reported in *Arabidopsis mutamts* that depending on the ratio of auxin and cytokinin, the organogenesis of roots and shoots is specified either with altered levels of hormones or altered signaling.

Acclimatization was determined to be highly successful through achieving higher rate of plantlets survivability in the hardening medium composed by garden soil, sand and vermicompost. Admixture of all these three components may offer condusive environment by providing proper nutrients, adequate aeration and required minerals respectively to the plantlets. In agreeing with this fact Chitra Devi and Narmatha Bai[34] reported that for a leguminaceae member, Desmodium motorium, combination of garden soil, sand and vermicompost is most reliable and effective for higher plantlet survivability.

In conclusion, an efficient protocol was developed for successful micropropagation and multiple plant regeneration of an important medicinal plant *A. caesia* L. In this study we reported the *in vitro* propagation from leaf segments by using various growth regulators. This standardized protocol can be used for rapid and large scale propagation.

Conflict of interest statement

We declare that we have no conflict of interest.

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