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AN EFFICIENT AND REPRODUCIBLE METHOD FOR *IN VITRO* PROPAGATION OF *CASSIA ALATA* L. – AN IMPORTANT WOODY MEDICINAL PLANT

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ABSTRACT

A rapid and efficient protocol for the large-scale propagation of an important woody medicinal plant, *Cassia alata* L., is described. Cotyledonary node (CN) obtained from 15 day-old aseptic seedling were used as an explant and their morphogenetic potential was tested on MS media with various concentrations (0.5–12.5 µM) of 6-benzyladenine (BA), Kinetin (Kn), and 2-isopentinyl adenine (2iP) alone or in combination with different concentrations (0.1 - 1.0 µM) of naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA). Among the single treatment, BA at an optimal concentration of 7.5 µM was most effective in inducing multiple shoots where 90% explants responded with an average shoot number (9.6 ± 0.3) and shoot length (1.7 ± 0.1 cm) after 6 weeks of culture. A better shoot differentiation and elongation was achieved in a combined treatment of BA (7.5 µM) and IBA (0.5 µM). Repeated subculturing of the explant in the fresh MS medium promoted a higher rate of shoot multiplication where 25.2 ± 0.9 well-grown healthy shoots with an average shoot length of 4.0 ± 0.0 cm were obtained by end of fourth subculture passage. Rooting was best induced in shoots excised from proliferated shoot cultures on MS medium augmented with an optimal concentration of 0.5 µM IBA. The in vitro-raised plantlets with well-developed shoots and roots were successfully established in thermocol cups containing soilrite and eventually transferred in garden soil with 84% survival rate. The results of this study provide the first report on *in vitro* plant regeneration of *Cassia alata*.

KEYWORDS: **Regeneration; Organogenesis; Subculturing; Rooting; Acclimatization.**

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INTRODUCTION

Plants are virtually inexhaustible source of biologically active compounds and extremely important in the lives of people throughout the world as they provide basic needs such as food, clothing, shelter and health care. They are also an important source of medicines and play a key role in world health[1]. There is a growing focus on the importance of medicinal plants and traditional health systems in solving the health care problems of the world. The age old traditional values attached with the various forest types and the varieties of forest products (i.e., medicinal plants) have gained tremendous importance in the present century [2]. The continuous exploitation of several medicinal plant species from the wild and substantial loss of their habitats during past 15 years[3] have resulted in declining the population of many high value medicinal plant species over the years. Information on the propagation of medicinal plants is available for less than 10% and agro-technology is available only for 1% of the total known plants globally[4]. Furthermore, in order to meet the ever growing demand of medicinal plants, *in vitro* farming of these plant species using biotechnological tools and techniques is imperative. Apart from meeting the present demand, *in vitro* culture using advanced biotechnological technique like plant tissue culture may help in conserving the wild genetic diversity of medicinal plants. Biotechnological tools are also equally important for multiplication and genetic enhancement of the medicinal plants for the production of secondary metabolite like quinine, rich in pharmaceutical properties.

Cassia alata L. (Fabaceae), commonly known as ringworm bush, is an erect woody medicinal shrub or small tree distributed mainly in the tropics and subtropics. The plant is a source of chrysoeriol, kaempferol, stearic acid, palmitic acid[5]. The leaves are reported to be useful in treating convulsion, syphilis, gonorrhoea, heart failure, abdominal pains, oedema, stomach problems, fever, asthma, snake bite and is also used as a purgative[6]. Besides, the plant extracts of this species has shown several pharmacological properties such as antibacterial[7], antifungal[8], antiseptic[9], anti-inflammatory[10], analgesic[11] and anti-hyperglycemic activities[12]. In addition, an important compound quinone having anti-malarial activity is also found in seeds and leaves which attracts recently on research in this plant[13]. The conventional method of propagation of *Cassia* species is via seed, however, propagation through seed is unreliable due to poor germination which restricts its propagation in natural conditions[14]. Alternative propagation methods would be beneficial in accelerating large scale multiplication, improvement and conservation of the plant. Tissue culture technology has often been successfully utilized for propagation of those plants where conventional methods have limitations or those which cannot be propagated by traditional propagation methods[15]. There are few reports on in vitro propagation of this species[16, 17]) which too yielded low number of shoots and cannot be useful for the large scale production and propagation. Therefore, there is a need to devise a method for the development of a large scale multiplication protocol for commercial production using different Plant Growth Regulators (PGRs).

MATERIALS AND METHODS

Establishment of aseptic seedlings

The seeds of *C. alata* were collected from the plant grown at the University. Seeds were washed thoroughly first under running tap water for 30 min to remove adherent particles, treated with a liquid detergent labolene (5% v/v) for 10 min followed by washing in tap water, and rinsed five times with double distilled water. These were surface sterilized for 10 min in 0.1% (w/v) $HgCl_2$ followed by repeated washes with sterile double distilled water. The seeds were kept for 2-3 days in a flask containing warm sterile double distilled water covered with cotton plug. The swelled seeds were then inoculated in MS medium[18] for germination. Cotyledonary nodes (CN) excised from 15-day-old aseptic seedlings was used as explants.

Culture media and conditions

The nutrient medium used in all the experiments consisted of MS salts and vitamins with 3% (w/v) sucrose (Qualigens Fine Chemicals, Mumbai, India). All the salts used were of analytical grade. The pH of the medium was adjusted to 5.8 using 1N NaOH. All the culture vials were incubated in culture room at 25 ± 2^0C under 16/8 h (light/dark) cycle with a light intensity of 50 µmol$m^{-2}s^{-1}$ supplied by 40W cool-white fluorescent tubes (Philips Electronics India Ltd.) and the relative humidity was maintained between 50-60%.

Shoot induction and multiplication

Cotyledonary nodes were placed on MS medium augmented with various cytokinins (BA, Kn and 2iP) at different concentrations (0.0, 0.5, 2.5, 5.0, 7.5, 10.0 and 12.5 µM) either singly or in combination with auxins (IBA, NAA, IAA) as listed in Table 1, 2, 3. The experiments were also designed to assess the effect of different initial pH values (5.0, 5.4, 5.8, 6.0 and 6.4). To induce shoot multiplication, growth, and elongation, subculturing onto the same medium was performed every 3 weeks. The multiplication potential of the explant was estimated by recording the data on number of shoots per explant and shoot length after 6 and 12 weeks of culture.

In vitro rooting

Healthy and well-elongated shoots (3–5 cm) were excised and transferred to rooting media composed of MS medium supplemented with different auxins (NAA, IBA) at various concentrations (0.0, 0.1, 0.5, 1.0 µM) as illustrated in table 4. Data on percentage of rooting, mean number of roots and root length per shoot were recorded after 4 weeks of transfer to rooting media.

Acclimatization

Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to thermocol cups containing sterile soilrite under diffused light (16:8 h photoperiod) conditions. Potted plantlets were covered with transparent polythene bags to ensure high humidity, and watered every 3 days for 2 weeks. Polythene bags were opened after 2 weeks in order to acclimatize plants to green house. After 4 weeks, the plants were transferred to pots containing normal garden soil, vermiculite and (1:1) vermiculite-garden soil mixture and maintained in a greenhouse under natural light.

Statistical analysis

All the experiments were repeated thrice with 20 explants for each treatment. The data obtained was analyzed using statistical software, SPSS Version 17 (SPSS Inc. Chicago, USA) and means were compared using Duncan's multiple range test (DMRT) at 5% level of significance. All the results were expressed in Mean ± Standard error.

RESULTS

Seed Germination

In vitro inoculated seeds germinated within 3 - 4 days on MS basal medium containing sucrose, recording 90% germination. Roots appeared after 3- 4 d and the cotyledonary leaves emerged after eighth day of culturing. All the shoots attained a height of 3-5 cm within 15 d of culturing, exhibiting a synchronized seed germination pattern. Cotyledonary node (CN) explants (Fig 1a inset) obtained from these seedlings were inoculated onto MS medium supplemented with different PGRs combinations for the induction of multiple shoots.

Effect of Cytokinin

CN explants grown on MS media devoid of any hormone failed to produce shoots, even after 6 weeks of culture and eventually necrosed, whereas the presence of growth regulators favored axillary bud initiation. The regeneration ability of cotyledonary node segment was promoted strongly with the application of growth regulators but in different morphogenic responses. The effect of various cytokinins (BA, Kn and 2iP) at different concentrations (0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0 µM) showed a marked effect on multiple shoot induction and plant regeneration from CN explants, and significant differences were observed on response expressed as shoot number and shoot length (Table 1). BA was found to be most effective than Kn and 2iP in respect to

initiation and subsequent proliferation of shoots (Table 1). Kn and 2iP showed almost similar type of morphogenetic response. The maximum percentage response (90%), highest mean number (18.3 ± 0.5) of shoots and maximum shoot length (3.4 ± 0.2 cm) were observed on a medium supplemented with 7.5 µM BA after 12 weeks of incubation (Table 1; Fig 1b). Thus, this concentration was considered to be the optimal concentration in inducing bud break and maximum shoot regeneration. When, Kn was used as the sole source of cytokinin, a slight basal callusing was observed with a low regeneration frequency (Table 1). A maximum of 3.6 ± 0.2 shoots with the highest percentage (89%) of responding explants was produced at 7.5 µM Kn after 6 weeks of incubation. However, in this case, the shoots exhibited a better elongation as compared to those induced using BA. In the medium augmented with 2-iP, comparatively few shoots were formed with a low regeneration frequency, the highest being 3.3 ± 0.2 shoots with 70% response at 7.5 µM concentration after 6 weeks of culture, but the elongation of regenerated shoots was maximum compared to BA or Kn (Table 1), indicating a tendency of the regenerated shoots towards apical dominance. When the concentration of each cytokinin was lowered, the number of shoots per explent was reduce.(10 µM), the number as well as the percent response was drastically reduced (Table 1).

Effect of auxin and cytokinin

Effect of combined treatments of cytokinins along with an auxin was evaluated for shoot multiplication rate by taking the optimized concentration of each cytokinins in combination with different concentrations (0.1, 0.5, 1.0 µM) of auxins (NAA, IAA, IBA) and the morphogenetic responses are summarized in Table 2. Inclusion of any of the auxins together with cytokinin improved all parameters evaluated. The number and frequency of shoot with regard to the growth regulators used in the medium also varied (Table 2). Presence of high cytokinin and low auxin positively influenced the induction of shoots. BA along with IBA exhibited a synergistic effect on multiple shoot formation where 90% response with 25.2 ± 0.9 shoots per explants, and the longest shoot length (4.0 ± 0.0) was observed on a medium containing BA (5.0 µM) ± IBA (0.5 µM) (Table 2; Fig 1c) after 12 weeks of culture. The percentage response (upto 90%) and the number of shoots (25.2 ± 0.9) per CN explant increased with an increasing concentration of IBA upto 0.5 µM. However, a gradual decrease in number of shoots per explant was observed at lower (0.1 µM) or higher concentration (1.0 µM) of IBA. Combinations of auxins along with 2iP (7.5 µM) and Kn (7.5 µM) also yielded favorable results and multiplication. Effectiveness of auxins for stimulating shoot proliferation synergistically with cytokinins (BA, Kn, and 2iP) follows the order NAA >IAA>IBA (Tables 2).

Effect of different basal media on shoot regeneration

Standardization of an appropriate culture medium with use of correct growth regulators is critical for optimum growth response of the explants. In the present investigation, the performance of different tissue culture media viz; ½MS, WPM, ½WPM, B5, ½B5 were tried in full and half strength medium, in which full strength medium supplemented with optimal concentration of BA (7.5 µM) + IBA (0.5 µM) proved best as compared to half strength media (Fig. 2) on multiple shoot induction and multiplication from cotyledonary node explants evaluated.

Effect of subculturing

In vitro raised shoots of *C. alata* got multiplied and proliferated onto the same medium. An average of 27–30 shoots and shoot length of 4 cm were obtained from CN explants on BA (7.5 µM) and IBA (0.5 µM) after first four culture passages, got stabilized at fifth passage (Fig. 2) and beyond which a gradual decline in multiplication rate was noticed. The results obtained confirm the positive effect of relatively high doses of BA (5.0 and 7.5 µM) on the production of shoots and axillary-buds up to the fourth subculture and the sharp drop in production thereafter (Fig. 3). At low doses (1.0 and 2.5 µM), the propagation of shoots buds varied considerably during the successive subcultures but always remained low.

In vitro rooting

Shoots regenerated from nodal explants failed to produce roots on MS basal medium without PGRs. Therefore, rooting of individual microshoots was made by transferring to MS basal medium containing different concentrations of NAA and IBA (Table 3). Two weeks after inoculation, root formation was noticed from the cut portion of the shoot. The isolated shoots when transferred to the rooting medium supplemented with NAA resulted in basal callusing within 12-14 days of incubation and induced thin, slender, shorter root in large number. Whereas the roots, obtained from regenerated shoots on MS medium containing IBA were thick, with secondary root hairs, which helped in establishing the plantlets in the soil (Fig 1d). Therefore, IBA was considered better for root induction without inducing callus. The highest number (7.1 ± 0.2) of roots per shoot was recorded at 0.5 µM IBA (Table 3).

Acclimatization

The period of transition during the process of hardening after transfer from the *in vitro* to the *ex vitro* environment is considered to be the most important step in tissue culture. One important factor during acclimatization is the type of potting material used. Among the four planting substrates tested (soilrite, garden soil, vermiculite and (1:1) vermiculite-garden soil mixture, the highest survival rate (84%) was achieved in soilrite (Fig 1e) whereas 76%, 72% and 64% plantlets survived in garden soil and vermiculite, vermiculite-garden soil mixture respectively after 4 weeks of transplantation (Table 4). There was no detectable variation among the potted plants with respect to morphological and growth characteristics.

Figure 1
Explanation of

a. Inset – excised cotyledonary explant
b. Shoot multiplication on BA(7.5 µM) + IBA (0.5 µM) after 6 weeks of culture
c. Shoot elongation and proliferation BA(7.5 µM) + NAA (0.5 µM) after 12 weeks of culture
d. Rooting of in vitro raised shoots on MS + IBA (0.5 µM)
e. An acclimatized plant in soilrite

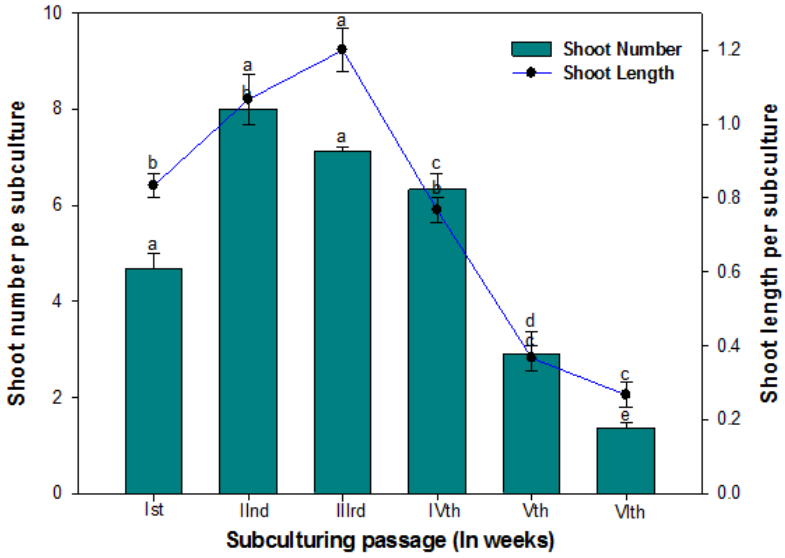

Figure 2

Effect of successive 3-week subculture passages on mean shoot numbers and shoot lengths per cotyledonary node explants of C. alata L. on 7.5 µM BA + 0.5 µM NAA. Bars represent the means ± SE. Bars denoted by the same letter within response variables are not significantly different (p= 0.05) using Duncan's multiple range tests.

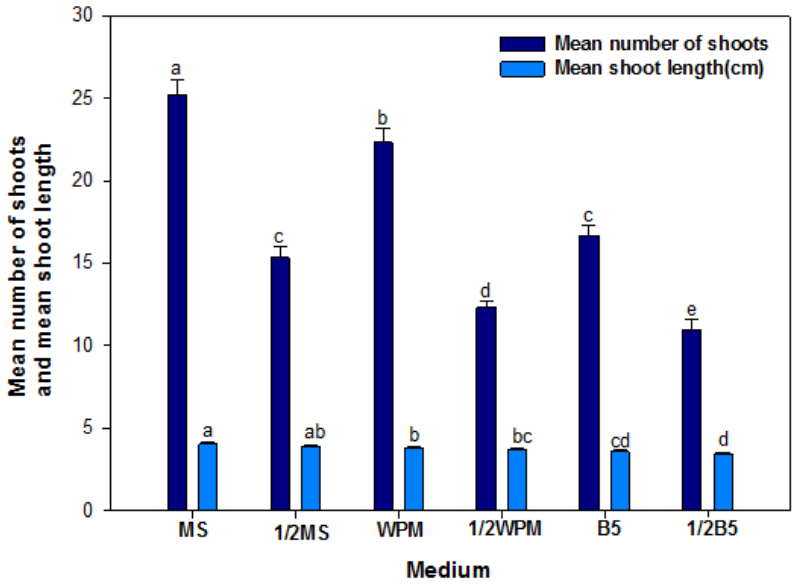

Figure 3

Effect of different basal medium on shoot regeneration from cotyledonary node explants of C. alata L. supplemented with BA (7.5 µM) + NAA (0.5 µM) after twelve weeks of culture. Bars represent the means ± SE. Bars denoted by the same letter within response variables are not significantly different (p= 0.05) using Duncan's multiple range tests.

Table 1

Effect of different concentrations of cytokinins on multiple shoot regeneration from cotyledonary node segments of Cassia alata in MS medium after 6 and 12 weeks of culture

PGRs(μM)		% Response	6 weeks		12 weeks	
BA 2iP	Kn		Mean no. of shoots per explant	Mean shoot length (cm)	Mean no. of shoots per explant	Mean shoot length (cm)
0.0 0.0	0.0	0	0.0 ± 0.0^{j}	0.0 ± 0.0^{j}	0.0 ± 0.0^{i}	0.0 ± 0.0^{g}
0.5		75	3.6 ± 0.3^{e}	1.0 ± 0.1^{h}	5.9 ± 0.2^{e}	2.12 ± 0.3^{f}
2.5		78	6.4 ± 0.5^{c}	1.5 ± 0.1^{fgh}	11.3 ± 0.7^{cd}	2.42 ± 0.2^{def}
5.0		84	8.2 ± 0.4^{b}	1.6 ± 0.2^{efg}	15.4 ± 0.1^{b}	3.24 ± 0.1^{abc}
7.5		90	9.6 ± 0.3^{a}	1.7 ± 0.1^{defg}	18.3 ± 0.5^{a}	3.4 ± 0.2^{abc}
10.0		85	8.6 ± 0.2^{b}	1.5 ± 0.2^{fgh}	12.2 ± 0.2^{c}	2.9 ± 0.2^{bcde}
12.5		80	5.5 ± 0.2^{d}	1.3 ± 0.0^{gh}	10.4 ± 0.4^{d}	2.8 ± 0.1^{cdef}
0.5		73	2.0 ± 0.3^{hi}	1.6 ± 0.2^{efg}	2.9 ± 0.3^{h}	2.3 ± 0.1^{ef}
2.5		75	2.2 ± 0.1^{ghi}	2.0 ± 0.1^{bcdef}	3.3 ± 0.4^{fgh}	2.8 ± 0.3^{bcdef}
5.0		82	2.9 ± 0.1^{efgh}	2.1 ± 0.1^{bcde}	4.4 ± 0.3^{f}	3.4 ± 0.2^{abc}
7.5		89	3.6 ± 0.2^{e}	2.7 ± 0.1^{a}	5.9 ± 0.1^{e}	3.9 ± 0.2^{a}
10.0		83	2.6 ± 0.2^{fgh}	2.4 ± 0.1^{abc}	4.5 ± 0.5^{f}	3.3 ± 0.1^{abc}
12.5		78	2.3 ± 0.3^{ghi}	1.9 ± 0.0^{cdefg}	3.3 ± 0.1^{fgh}	3.0 ± 0.2^{bcde}
0.5		72	1.5 ± 0.1^{i}	2.0 ± 0.0^{bcdef}	2.6 ± 0.1^{h}	2.8 ± 0.0^{cdef}
2.5		74	2.0 ± 0.1^{ghi}	2.2 ± 0.2^{abcd}	3.0 ± 0.1^{gh}	3.2 ± 0.4^{abcd}
5.0		80	2.4 ± 0.1^{fgh}	2.4 ± 0.2^{abc}	3.5 ± 0.1^{fgh}	3.4 ± 0.3^{abc}
7.5		84	3.3 ± 0.2^{ef}	2.7 ± 0.1^{a}	5.8 ± 0.5^{e}	4.0 ± 0.3^{a}
10.0		82	3.0 ± 0.1^{efg}	2.5 ± 0.1^{ab}	4.4 ± 0.2^{f}	3.6 ± 0.1^{ab}
12.5		74	2.1 ± 0.1^{ghi}	2.4 ± 0.1^{abc}	4.2 ± 0.1^{fg}	3.5 ± 0.1^{abc}

Values represent means ± standard error of 5 randomly selected readings of 20 replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.

Table 2

Effect of different concentrations of cytokinins and auxins on multiple shoot regeneration from cotyledonary node segments of C. alata in MS medium after 12 weeks of culture

BA	Kin	2iP	IBA	NAA	IAA	% Response	Mean no. of shoots per explant	Mean shoot length (cm)
7.5			0.1			88	18.9 ± 0.1^{d}	3.5 ± 0.1^{ij}
7.5			0.5			89	20.3 ± 0.4^{c}	3.9 ± 0.9^{ghij}
7.5			1.0			87	19.2 ± 0.0^{d}	3.4 ± 0.1^{j}
7.5				0.1		89	24.3 ± 0.6^{ab}	3.6 ± 0.0^{hij}
7.5				0.5		92	25.2 ± 0.9^{a}	4.0 ± 0.0^{defg}
7.5				1.0		90	23.6 ± 0.2^{b}	3.6 ± 0.1^{hij}
7.5					0.1	86	19.0 ± 0.0^{d}	3.7 ± 0.1^{ghij}
7.5					0.5	89	20.7 ± 0.8^{c}	3.9 ± 0.0^{efgh}
7.5					1.0	85	19.2 ± 0.0^{d}	3.4 ± 0.0^{j}
	7.5		0.1			86	6.2 ± 0.0^{efg}	4.1 ± 0.0^{bcdef}
	7.5		0.5			88	7.0 ± 0.0^{ef}	4.2 ± 0.1^{bcdef}
	7.5		1.0			85	6.0 ± 0.0^{fg}	4.0 ± 0.1^{efg}
	7.5			0.1		86	6.1 ± 0.1^{fg}	4.2 ± 0.1^{bcdef}
	7.5			0.5		90	7.2 ± 0.1^{e}	4.4 ± 0.1^{ab}
	7.5			1.0		85	6.0 ± 0.0^{fg}	4.1 ± 0.0^{bcdef}
	7.5				0.1	84	6.0 ± 0.1^{fg}	4.0 ± 0.0^{cdef}
	7.5				0.5	88	6.4 ± 0.1^{ef}	4.2 ± 0.1^{bcdef}
	7.5				1.0	83	5.9 ± 0.0^{fg}	4.0 ± 0.0^{cdef}
		7.5	0.1			85	6.0 ± 0.1^{fg}	4.1 ± 0.0^{bcdef}
		7.5	0.5			86	6.5 ± 0.1^{ef}	4.4 ± 0.1^{abcd}
		7.5	1.0			83	5.1 ± 0.7^{g}	4.2 ± 0.1^{bcdef}
		7.5		0.1		86	6.0 ± 0.1^{fg}	4.3 ± 0.1^{abcde}
		7.5		0.5		88	6.6 ± 0.2^{ef}	4.6 ± 0.1^{a}
		7.5		1.0		85	5.8 ± 0.0^{fg}	4.2 ± 0.1^{abcdef}
		7.5			0.1	83	6.2 ± 0.1^{efg}	4.3 ± 0.0^{abcde}
		7.5			0.5	86	6.4 ± 0.2^{ef}	4.4 ± 0.2^{abc}
		7.5			1.0	85	5.9 ± 0.1^{fg}	4.1 ± 0.0^{bcdef}

Values represent means ± standard error of 5 randomly selected readings of 20 replicates per treatment in three repeated experiments. Means sharing the same letter are not significantly different (P = 0.05) using Duncan's multiple range test.

Table 3
Effect of auxins on root induction from in vitro-raised microshoots of Cassia alata in MS medium after 4 weeks of culture

Auxins IBA NAA	% Response	Mean no. of roots/shoot	Mean root length/shoot (cm)
0.0 0.0	0	0.0 ± 0.0^{g}	0.0 ± 0.0^{e}
0.1	98	4.4 ± 0.1^{e}	2.6 ± 0.1^{d}
0.5	99	7.1 ± 0.2^{d}	3.4 ± 0.2^{abc}
1.0	97	5.0 ± 0.3^{e}	3.0 ± 0.1^{bcd}
2.0	96	3.6 ± 0.3^{f}	2.9 ± 0.1^{cd}
0.1	99	7.1 ± 0.2^{d}	3.2 ± 0.1^{bcd}
0.5	100	8.7 ± 0.2^{c}	3.8 ± 0.2^{a}
1.0	99	9.8 ± 0.0^{b}	3.5 ± 0.2^{ab}
2.0	98	12.7 ± 0.2^{a}	3.2 ± 0.2^{bcd}

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P= 0.05) using Duncan's multiple range test.
**** Significant at P = 0.05***

DISCUSSION

Plant hormones play an important role in many aspects of growth and development including dormancy, which may also be hormonally controlled. Cytokinins were reported to play a key role in DNA synthesis and cell division, which might be the reason for induction of multiple shoots. Different cytokinins generally express different activities in affecting axillary shoot formation *in vitro*[19]. CN explants of *Cassia alata* failed to develop shoot buds in growth regulator–free medium. In contrast, when the same explants were grown on culture media containing cytokinin, axillary shoots developed precociously, which proliferated to form clusters of secondary and tertiary shoots. Multiple shoot emergences from CN explants were observed on MS medium containing BA at different concentrations. The role of BA in multiple shoot formation from CN explants has been reported in many plants such as *Ricinus communis*[20], *Cassia siamea*[21], *Bauhinia tomentosa*[22], *Bauhinia racemosa*[23]. BA at the concentration of 7.5 µM was found optimal for maximum shoot induction. The increase in concentration of BA beyond optimal level had a negative effect, induction of callus from basal cut end was very much pronounced, which might be one of the reason for reduction of multiple shoot regeneration. Inhibitory effect of higher concentrations of BA on shoot multiplication has also been reported in other plant species such as *Rauvolfia tetraphylla*[24], *Vitex negundo*[25],*Cassia siamea*[21]. CN explants was responsive after 3 weeks in culture on MS medium containing Kn and 2iP with 72-89% response, whereas high (75–90%) response was exhibited by the use of BA. Thus, all cytokinins (BA, Kn and 2iP) were capable of shoot induction in CN segments, and BA was found to be significantly more effective than other cytokinins. Similar observations with different cytokinins have been reported in other plant species such as *Cassia siamea*[21], *Bauhinia tomentosa*[22]. The combination of various auxins (IAA, IBA and NAA) with the optimal cytokinin concentration was also studied for their ability to affect the shoot induction and multiplication rate. The synergistic effect of BA in combination with an auxin has been demonstrated in many medicinal plants like *Cassia siamea*[21], *Salix terasperma*[26], *Cassia angustifolia*[27]. In accordance with these reports, the present study also exemplifies the positive modification of shoot induction efficacy obtained by employing a low concentration of auxin in combination with a cytokinin. This differential morphogenetic response may be due to apical dominance which is controlled by the ratio of auxin and cytokinin and widely recognized to be caused by the action of basipetally transported auxin from apex and its consequent inhibition of axillary bud

growth[28]. It is clearly documented that cytokinin regulates auxin levels and vice versa. Both regeneration frequency and average number of shoots produced per explants were enhanced significantly when compared with single cytokinin only. All the auxins were capable of inducing more than 70% explants to respond positively, but the combination of BA (7.5 µM) with IBA (0.5 µM) was found to be the best in *C. alata*. Such stimulating effects of combinations of these two hormones are well supported by earlier studies on *Melia azeadrach*[29] and *Ficus anastasia* [30], *Pyrus pyrifolia*[31]. Thus, individual and interactive effect of BA and IBA could ensure better *in vitro* regeneration, and their synergism in proper concentration was extremely favorable for multiplication. However, in *Psoralea corylifolia*[32] and *Vitex negundo*[25], the best combination was observed on a medium containing BA and NAA, whereas IAA and BA combination was the optimum in *Chonemorpha grandiflora*[33]. Almost similar response was observed with BA and IBA or NAA combination in case of *Pogostemon heyneanus*[34]. The promoting effect of Kn and an auxin (IBA, NAA, IAA) combination on proliferation of axillary buds showed synergistic effect on shoot multiplication and elongation. Such stimulating effects of culture response on a medium containing Kn and IBA, NAA and IAA has been observed in *Chlorophytum arundinaceum*[35]; *Teucrium stocksianum*[36]. These results support the findings that the interaction of auxin and cytokinin is necessary for *in vitro* organogenesis, and high concentration of cytokinin and low concentration of auxin appeared to be a prerequisite for differentiation of shoot buds.

The highest rate of micropropagation will often depend not only on the selection of the most suitable explants, but also on the best basal medium for that tissue[37] (Martin 2004). The nutritional requirement varies according to the cells, tissues, organ and protoplast and also with respect to particular plant species. The best basal medium for the highest multiple shoot induction and multiplication in *Cassia alata* from cotyledonary node explant was found to be full strength MS medium while lower shoot multiplication was observed in B5 basal medium. Similar results have also been reported in some woody plants including *Albizzia lebbeck* [38], *Rauvolfia tetraphylla* [39]. In many plant species, micropropagation requires two media– propagation and shoot elongation medium– making the micropropagation procedures cumbersome and uneconomical[40]. The effects of subculture on the multiplication rates achieved in culture are known to differ from one species to another. Shoot multiplication rate was found to be highest upto the fourth subculturing passages after which the shoot number decreased. Similar effect of subculturing was observed in *Bacopa monniera* where shoot induction and multiplication increased up to the third subculture passage, after which the frequency and number of shoots decreased[41]. In *Cassia angustifolia*, shoot production increased during subculturing and stabilized in the fifth passage[42]. The increase shoot number, due to repeated transfer of the mother explants, may be due to suppression of apical dominance during subculture that induced basal dominant meristematic cells to form new shoots [43]. Thus, the regenerating medium (MS) containing BA (7.5 µM) and IBA (0.5 µM) may be recommended as the best suitable medium for long-term maintenance of regenerative potential of CN explants of *C. alata.* During micropropagation, induction of rooting is an important step and losses at this stage have vast economic consequences [44]. Thus, research on adventitious root formation is highly important from the practical point of view. Response of rooting was very much dependent on type and concentration of auxins supplement in the media. Adventitious root induction in isolated micro-cutting of *Cassia alata* was achieved in the presence of various auxins (IBA and NAA) in MS medium. The equimolar concentration (0.5 µM) NAA and IBA induced maximum rooting response. Similar results were reported in *Garcinia indica* [45] and *Rauvolfia tetraphylla*[32]. Rooting in *in vitro* regenerated microshoots started after 2 weeks of incubation on MS medium supplemented with NAA with (98%) rooting frequency and the number of roots was also higher in comparison with IBA however the roots formed were slender and thin which is

unfavourable for acclimatization whereas the roots formed from shoots using IBA were thick with 99% rooting frequency. Therefore it was considered to be best rooting hormone for this species. The superiority of IBA as an effective rooting hormone is very well discussed in *Mucuna pruriens* [46], *Citrullus colocynthis*[47] and *Cassia angustifolia*[27].

The most critical and important step of micropropagation studies is the transfer of regenerants from artificial to natural environment. Plantlets with well-developed root and shoot system were successfully hardened off inside the growth room with different planting substrate. Soilrite being more porous substrate holds more water than vermiculite, vermiculite-garden soil mixture (1:1) and garden soil, and thus promoted better growth of tender roots of tissue culture raised plants during hardening. Similar observations were observed in *Tecomella undulate* [48]. The micropropagated plants showed uniform morphology and growth when compared with naturally grown plants. The protocol described in this study for regeneration of *C. alata* using cotyledonary explants of axenic seedlings is reproducible and improved method which could be useful for conservation and large scale planting of this economically and medicinally important woody shrub.

ABBREVIATIONS

BA 6-benzyladenine
Kn Kinetin
2iP 2-isopentenyl adenine
IBA Indole -3-butyric acid
NAA α- naphthalene acetic acid
IAA Indole-3-acetic acid
PGRs Plant Growth Regulators
MS Murashige and Skoog's medium

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