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In Vitro Propagation of *Cassia alata L* and Its Secondary Metabolites

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Abstract
Cassia alata (L.) *is traditionally used to treat various fungal infections, traditionally used as a remedy for poisonous bites. The objectives of the present study were to conservation of Cassia alata through the plant tissue culture and comparative of its secondary metabolite with field cultivated plant as well as in vitro. In micropropagation, multiple shoots were induced by the nodal region of the plant on Murashige and Skoog medium containing plant growth regulators such as 6-benzylaminopurine, Kinetin, and indole-3-acetic acid. The multiplication were achieved in the combination of 6-benzylaminopurine with naphthalene acetic acid or indole-3-acetic acid. The present investigation has observed that maximum number and shoots per explants was obtained on MS medium with 6-benzylaminopurine at 1.0 mg/l concentration and the roots were proliferated in the combination of 6-benzylaminopurine (1.0 mg/l) and indole-3-acetic acid (1.0 mg/L) concentration. Root induction was achieved on MS medium supplemented with IAA. The well-rooted in vitro propagated Cassia alata was successfully transferred to the greenhouse for acclimatization. Field cultivated plant was propagated through seedlings in sand. A 5 month propagated plant leafs were successfully collected for comparative study of total phenol and total flavonoids content. In the present secondary metabolite estimation has confirmed that total flavonoids (48.52 ± 0.79) was found to be maximum in micropropagated plant and total phenol content (50.0 ± 1.7) was found to be maximum in the field cultivated plant.*

Keywords: *6-benzylaminopurine, comparative, Murashige and Skoog medium, phenol, flavonoids, propagation*

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INTRODUCTION

The development of the new biotechnological way has opened up new areas for valuable medicinal plant conservation through micropropagation and genetic improvement. Plant cell culture result shows that higher rate of frequency of variation in regenerated plants [1]. In the present scenario, plant tissue culture has direct commercial importance and its application in basic sciences such as cell biology, genetics, biochemistry, and biotechnology are an evidence for its usefulness and applications [2]. *In vitro* micro propagation technique provides many advantages over conventional propagation methods. Micropropagation through shoot tip culture, often utilized to maintain clonal fidelity would be a special advantage in this technique [3]. *In vitro* regeneration and propagation is an efficient method to conserve biodiversity [4]. Reestablishment of the plant through *in vitro* culture is most effective for medicinal plants on mass scale from their natural habitats [5]. Plant tissue culture not only provides a new method for mass propagation but also makes possible for the production of disease-free and genetically modified plants. It is also provides a way for the production of important secondary metabolites [6, 7].

Plant improvement strategies in the past two decade is widening remarkably. Researchers identifies the diversification in the field of morphology, physiology, and genetic nature of the plants offers incredible opportunities for the identification, isolation, and characterization of new genes as well as to understand basic

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linkages among diverse plant kingdom. The angiosperms are the largest plant group consisting of plants with extensive assortment and multiplicity reported [8]. Plant tissue culture is a collection of techniques used to maintain or to grow the plant cells, tissues, or organs under sterile conditions on a nutrient culture medium of known composition. The small organs or pieces of tissue that are used are called explants [9]. Remedies derived from plants are still being used worldwide in the more traditional medicine system for the treatment of a various range of diseases and ailments including tuberculosis reported [10].

Micropropagation is, therefore, imperative to establish genetic uniformity of micropropagated plants and to confirm the quality of the plantlets for its commercial usage [11]. The presence or absence of variations during *in vitro* propagation depends upon the source of explants and the method of regeneration [12]. Our group has conducted much research into this particular area of micropropagation of plants with medicinal and ornamental value for conservation purposes. Some of the studies reveals that successful in several genera, including *Crinum* [13], *Tulbaghia* [14], *Scilla* [15], *Cyrtanthus, Eulophia* [16], *Eucomis* [17], *Hypoxis* [18], *Sandersonia* [19], *Gloriosa* [20], *Podocarpus* [21], *Cussonia* [22], *Emelia zeylanica* [23], and *Salvia* [24]. Plants play a vital role in traditional medicine around the world and it is also provides the crucial contribution to the pharmaceutical industry [25]. About 40% or more of the pharmaceuticals currently used in Western countries are fully or partially derived from natural resources reported [26]. Due to the growing commercial importance of *Cassia* secondary metabolites, there is great interest in enhancing their production through biotechnology. Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants [27]. *In vitro* plant cell cultures offer the possibility of obtaining desirable medicinal compounds as well as ensuring sustainable conservation and rational utilization of biodiversity.

Cassia alata belongs to the family Fabaceae. *Cassia alata* contains more flavonoids, anthraquinones, and polysaccharides and it has been reported to have many medicinal values [28]. The major active pharmacological compounds anthraquinone glycosides, which are responsible for laxative properties, it is reported [29]. *Cassia alata* is traditionally used as a remedy for poisonous bites and venereal infections. It exhibits antimicrobial activity [30]. The objectives of the present study were to micropropagate *Cassia alata* Linn. through *in vitro* protocol and to compare the total phenol and total flavonoids with field cultivated plant.

MATERIALS AND METHODS

Plant Material

In the presence study, *Cassia alata* plant was collected from the Botanical garden (11°21′45.1″North and 77°49′34.9″East) of K. S. Rangasamy College of Technology, Tiruchengode, Tamil Nadu, India. The young nodel plant material was collected approximately 1.5 cm in length using sterile blade. The collected plant material was dipped in water to prevent dehydration and cell damage.

Hormone Preparation

Auxins: 10 mg of auxins (IAA and NAA) were dissolved in few drops of 1N NaOH and finally made upto 10 ml by using sterile distilled water and used for media preparation.

Cytokinins: 10 mg of cytokinins (BAP and KIN) were dissolved in few drops of 1N HCl and finally made upto 10 ml by using sterile distilled water and used for media preparation.

Culture conditions: Single disinfected stem segments were cultured on MS medium [31] augmented with 6-benzylaminopurine (BAP) or kinetin (Kin) or indole-3-acetic acid (IAA) and in combination of BAP with naphthalene acetic acid or indole-3-acetic acid were prepared for the present study. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1N NaOH or 1N HCl before gelling with 0.8% (w/v) agar (Himedia). In all the experiments, the chemicals used were of analytical grade (Himedia, Qualigens, Merkard, and Sigma). The medium was dispensed into culture vessels (Borosil, Mumbai, India) and

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autoclaved at 105 kPa and 121°C for 15 minutes. The surface disinfected explants were implanted horizontally on the culture medium test tubes (150×25) containing 50 ml medium and plugged tightly with nonabsorbent cotton. All the cultures were incubated at 25+ 2°C under 16 h photoperiod of 45 to 50 mol m^{-2} s^{-2} irradiance provided by the cool white fluorescent tubes (Philips, India) and with 55% to 60% relative humidity. All subcultures were done at 3-week intervals.

Sterilization of the Explants

The selected fresh explants were thoroughly washed with tap water for 20 minutes and surface sterilization done by using detergent solution and subsequently followed by tween 20 for 5 minutes and thoroughly washed with distilled water to remove the surface contaminants. In the laminar air chamber, the selected explants was treated with 0.1% of Mercuric chloride for one minute, 70% ethanol for 1 minute, and finally the selected explants was washed with 0.5% antifungal solution for 10 minutes. The explants were thoroughly rinsed routinely with sterilized distilled water for 5 times and used for further analysis of studies.

Micropropagation

The explants were successfully collected from the healthy plant of *Cassia alata*. Shoots were successfully sterilized. After sterilization the explants are successfully inoculated in the prepared MS medium supplemented with various concentration and combination of auxin and cytokinin.

Maintenance of the Culture

After sterilization, the explants were inoculated in the medium under aseptic condition. The cultures were maintained at 25°C ± 2°C with 10 hours light period and 50% to 60% humidity.

Rooting and Acclimatization of Plantlets

The rooted plantlets were removed from the culture tubes, washed thoroughly with tap water to remove traces of agar, treated with 0.3% bavistin and planted in small pots filled with a mixture of garden soil, sand, and farmyard manure (2:1:1). Plants were maintained in a growth chamber. They were acclimatized to a reduced relative humidity by gradual opening of the plastic cover, and after 3 weeks they were completely uncovered and hardened to greenhouse conditions.

Field Cultivation and Collection

Cassia alata seeds were allowed to germination in sand. The seeds were germinated on 9th day. The plants were grown up at 5-meter tall. The explants were collected from that plant for analysis.

Total Phenolic Estimation

In vitro hardened plant and field cultivated plant leafs were collected for total phenol estimation. Leafs extracted were collected using aqueous solution. The total phenolic contents of the leaf samples were measured using a modified colorimetric Folin-Ciocalteu method. A volume of 0.5 mL of deionized water and 0.125 mL of a known dilution of the extract were added to a test tube. Folin-Ciocalteu reagent (0.125 mL) was added to the solution and allowed to react for 6 min. Then, 1.25 mL of 7% sodium carbonate solution was aliquot into the test tubes, and the mixture was diluted to 3 mL with deionized water. The colour developed for 90 minutes, and the absorbance was read at 760 nm using a UV spectrophotometer.

Total Flavonoids Estimation

The flavonoid content of the apple samples was measured using a modified colorimetric method. A volume of 0.25 mL of a known dilution of extract was added to a test tube containing 1.25 mL of distilled water. To the mixture was added 0.075 mL of 5% sodium nitrite solution, and this was allowed to stand for 5 minutes. Then, 0.15 mL of 10% aluminium chloride was added. After 6 minutes, 0.5 mL of 1 M sodium hydroxide was added, and the mixture was diluted with another 0.275 mL of distilled water. The absorbance of the mixture at 510 nm was measured immediately using UV spectrophotometer.

RESULTS AND DISCUSSION

***In Vitro* Propagation**

Collected nodal explants were cultured in MS medium with different concentration of BAP, IAA, KIN, and then combination of BAP

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(1.0mg/L) with IAA and NAA. The nodal explants shoots were elongated for the multiple shoot development. BAP 1.0 mg/ L was found to be maximum in response percentage (100), shoot elongation (4.60 ± 1.020) and number of shoots sprouting (7.20 ± 0.750). Kinetin 1.5 mg/ L was found to be maximum in response percentage (80), shoot elongation (3.57 ± 0.530), and numbers of shoots sprouting (4.50 ± 1.110;).

MS with Kinetin (0.50 mg/L) or (1.50 mg/L) was observed same percentage of response. These concentration were compared with number of shoot formation and shoot elongation, it was confirmed to be MS with Kin (1.50 mg/L) shows better result. A similar result was found in *Dalbergia sisso* from callus derived from semi-mature zygotic embryos [32] (Table 1).

Fig. 1 (A): *Multiplication of shoot obtained on MS medium + 1.0 mg/L BAP (B) Multiplication of shoot and root obtained in MS + 1.0 mg/L BAP + 1.0 mg/L IAA.*

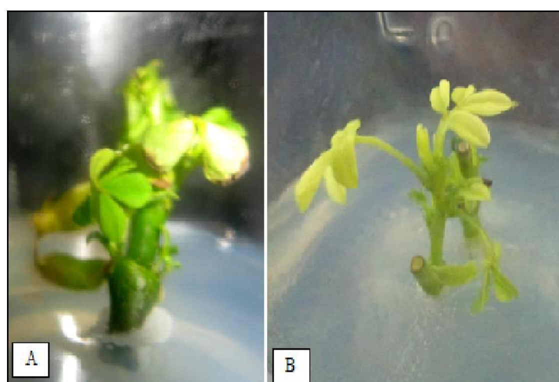

Fig. 2 (A): *Multiplication of shoot obtained on MS medium + 0.50 mg/L Kin (B) Multiplication of shoot obtained in MS + 1.5 mg/L Kin.*

IAA 1.5 mg/ L was found to be maximum in response percentage (80), shoot elongation (6.00 ± 0.440) and numbers of shoots sprouting (5.50 ± 1.110). MS with IAA (0.05 mg/L) or (0.50 mg/L) or (1.50 mg/L) was observed same percentage of response. These concentration were compared with number of shoot formation and shoot elongation, it was confirmed to be MS with IAA (0.50 mg/L) shows better result in *Cassia alata*.

When compared with Kinetin and IAA, BAP shows maximum response percentage, high number of shoots sprouts and maximum shoot length. From that criteria BAP (1.0 mg/L) was selected for different combination and concentration of IAA and NAA. The Kinetin hormone having relatively low shoot regeneration and Kinetin hormone regenerate few shoots is reported (Figures 1–4).

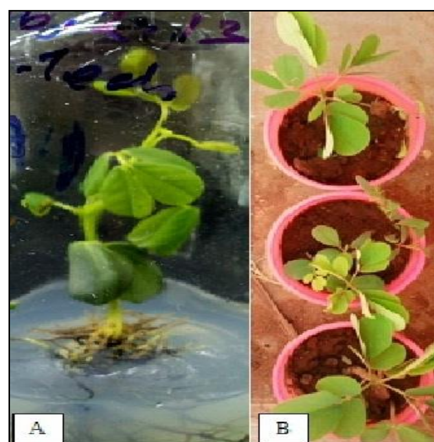

Fig. 3 (A): *Maximum shoot length obtained for BAP 1.0 mg/L (B) Plantlet grows in Green house shed for hardening.*

Fig. 4: *Multiplication obtained from 20th day of subculture in MS + BAP (1.0 mg/L).*

Table 1: *Effect of BAP, KIN and IAA Concentrations on the Percentage of Reactive Explants, the Number of Shoots and Maximum Length of the Shoot Per Explants After 30 Days of the Culture on MS Medium.*

Plant growth regulators	Concentration of hormone (mg/L)	Response percentage	Number of shoots per explants	Shoot length per explants (cm)
Control	—	100	2.00 ± 0.630	1.80 ± 0.400
BAP	0.05	60	2.33 ± 0.470	2.10 ± 0.140
	0.50	80	4.50 ± 0.860	2.62 ± 0.410
	1.00	100	7.20 ± 0.750	4.60 ± 1.020
	1.50	80	3.00 ± 0.710	3.63 ± 0.410
KIN	0.05	60	3.33 ± 0.940	2.73 ± 0.205
	0.50	80	2.75 ± 0.830	2.07 ± 0.376
	1.00	60	2.66 ± 0.940	2.10 ± 0.535
	1.50	80	4.50 ± 1.110	3.57 ± 0.530
IAA	0.05	80	2.50 ± 0.500	2.47 ± 0.390
	0.50	80	5.50 ± 1.110	6.00 ± 0.440
	1.00	60	2.66 ± 0.940	2.30 ± 0.700
	1.50	80	3.75 ± 0.829	3.13 ± 0.980

Table 2: *Effect of IAA and NAA Combination with BAP for the Explants, the Number of Shoots and Maximum Length of the Shoot Per Explants After 30 Days of the Culture on MS Medium.*

Plant growth regulators	Concentration of Hormone (mg/L)	Response percentage	Number of shoots per explants	Shoot length per explants (cm)
BAP + IAA	1.0 + 0.05	80	2.75 ± 0.830	2.25 ± 0.540
	1.0 + 0.50	60	3.33 ± 0.471	2.50 ± 0.454
	1.0 + 1.00	100	6.80 ± 0.748	3.58 ± 0.490
	1.0 + 1.50	80	4.00 ± 0.700	3.22 ± 0.280
BAP + NAA	1.0 + 0.05	80	2.25 ± 0.433	2.67 ± 0.200
	1.0 + 0.50	100	6.00 ± 0.630	3.28 ± 0.270
	1.0 + 1.00	60	4.66 ± 0.471	2.90 ± 0.290
	1.0 + 1.50	80	3.25 ± 0.829	2.62 ± 0.260

Effect of Hormone Combination

The maximum response percentage was found to be that MS with combination of BAP (1.0 mg/L) with IAA (1.0 mg/L) followed by maximum shoot elongation (3.58 ± 0.490) and numbers of shoots sprouting (6.80 ± 0.748).

MS with combination of BAP (1.0 mg/L) and IAA (0.05mg/L) or IAA (1.50mg/L) was observed same percentage of response (80). It confirms that decreasing trend in response percentage could be observed with increase in the IAA concentration. The maximum response percentage was found to be that MS with combination of BAP (1.0 mg/L) with NAA (0.50 mg/L) followed by maximum shoot elongation (3.28 ± 0.270) and numbers of shoots sprouting (6.00 ± 0.630).

MS with combination of BAP (1.0 mg/L) and NAA (0.05mg/L) or NAA (1.50 mg/L) was observed same percentage of response (80). In the present investigation, BAP (1.0 mg/L) was found to be superiority hormone concentration for *Cassia alata*. Kinetin and IAA was failed to response maximum length of shoot and sprout formation for shoot induction. In combination of BAP with IAA and NAA shows maximum response percentage in IAA (1.0 mg/L; Table 2).

BAP plays an important role for shoot regeneration has been already reported. Cytokinins are mainly involved for the regeneration of shoot bud. Then the combination of auxin and cytokinin in different proportion are very difficult to judge

the induction of shoot and root in many species is reported.

Plants with secondary metabolites have been used by humans to treat infections, health disorders, and illness [33]. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, bio-based fuels and plastics, enzymes, preservatives, cosmetics (cosmeceuticals), natural pigment, and bioactive compounds. Secondary metabolites derived from plant cell culture can be generated on a continuous basis and there are no seasonal constraints for the production. The interests in phenolic compounds, particularly flavonoids has considerably increased in recent years because of their broad spectrum of chemical and diverse biological properties. The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses [34].

Antioxidants have been shown to play an important role in preventing many diseases like cancer, inflammation, and brain dysfunction and plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications and fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products. Antioxidants are important species which possess the ability of protecting organisms from damage caused by free radical-induced oxidative stress. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators [35]. Also, many other plant species have been investigated in the search for novel antioxidant but generally there is still a demand to find more information concerning the antioxidant potential of plant species [36].

Qualitative and quantitative analyses of secondary metabolites are advantageous for the micropropagation of medicinal plants and it has been reported in many medicinal plant species [37,38]. Flavonoids have been associated with possible role in the prevention of several chronic diseases involving oxidative stress as well as their protective effect against low-density lipoprotein oxidation [39]. It is group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, and anti-inflammatory action. Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity [40]. Flavonoids are known for their free radical scavenging and anti-oxidant activities, the positions of the substituents also affect the physiological properties of different flavonoids. It is most important pigments for flower coloration producing yellow or red/blue pigmentation in petals and those colors are a mean to attract pollinator animals [41]. Over 5,000 naturally occurring flavonoids have been characterized from various plants [42].

Among the various concentration of the hormones and combinations, BAP has shows that best response in terms of multiple shoot generation. The better multiple shoots were induced from intermodal explants after 30 days of culture on MS medium in 1.0 mg/L of the BAP. Regarding the interaction between the combinations and concentrations of hormones in *Cassia alata* shows maximum growth was observed in BAP (1.0 mg/L) and IAA (1.0 mg/L) and the roots were induced in the indole acetic acid with BAP hormone [43,44]. Obtained highest rooting by using IBA but IAA shows highest rooting percentage is reported by [45].

For acclimatization, the well-developed rooted plantlets are gently removed from the cultured medium. Agar which is present in the roots are washed off. *In vitro* plantlets were transferred to pots containing a mixture of soil, vermiculate, and sand (2:1:1) and healthy roots appeared after 2 weeks. Initially, the plants are covered with polyethylene bags to maintain the humidity during first 1 week and watered regularly. The survival rate of the clones was about 95%. The plants were hardened for 10 to 15 days before being transferred to the greenhouse. The plants are transferred to the green house for acclimatization. The essential system of micropropagation is successful after transferring the plantlets from tissue culture vessels to ambient condition found *ex vitro.*

Comparative Studies of Secondary Metabolite

After 5 months from seed germination, leafs were collected for estimation of total phenol and flavonoids. Estimated results were mentioned in Table 3.

Table 3: *Total Phenolic and Flavonoids Content of Aqueous Extract of Cassia alata L.*

Type	Total phenolic content (mg /L)	Total flavonoids content (mg/L)
In vitro cultivated	15.68 ± 1.60	48.52 ± 0.79
Field cultivated	50.0 ± 1.7	8.72 ± 0.92

Total phenolic content was found to be maximum, whereas total flavonoids were found to be low in field cultivated plant. Total flavonoids was found to be maximum followed by total phenolic content was found to be very low for *in vitro* raised plant. Its shows that *in vitro* raised plant have high medicinal value because it was observed to maximum content of flavonoids content. It was known that *in vitro* culture system is a much more attractive alternative than directly plant raised from seed for the production of high value secondary metabolites, but depending upon plant species metabolites content may vary [46].

CONCLUSION

The present investigation has confirmed that BAP (1.0 mg/L) was found to be the best response percentage in *Cassia alata*. The maximum response percentage was 100%. Only BAP (1.0 mg/l) with MS medium yields maximum number of shoots (7.20 ± 0.750) and high shoot elongation (4.60 ± 1.020).

Roots were induced in MS medium with the combination of BAP (1.0 mg/L) and IAA (1.0 mg/L). It was the maximum response percentage (100) in combination of hormones. It must be noted that the number of shoots and shoot length was higher in case of MS with BAP (1.0 mg/L) cultured explants.

Kinetin and Indole-3-acetic acid were failed to response in maximum percentage, number of shoots formation, and shoot elongation in *Cassia alata.* It concludes that cultured explants of *Cassia alata* in MS + BAP (1.0 mg/L) exhibited superiority over cultured explants in MS + Kin or MS + IAA. Based on these observations we propose an efficient protocol to micropropagate the *Cassia alata* from explants of the seedlings.

Total phenolic content was found to be low and flavonoids content was found to be high in *in vitro* cultured plants. Despite, the immense variety of flavonoids content was estimated from *in vitro* cultured plant. Its shows *in vitro* propagated plant had high medicinal value.

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