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In-vitro Regeneration from Direct and Indirect Organogenesis of Crescentia alata Kunth an Important Multipurpose Medicinal Tree

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

In this present work, an in-vitro regeneration protocol for Crescentia alata (C. alata) was developed using various explants on Murashige and Skoog (MS) medium augmented with different concentrations and combinations of plant growth regulators (PGRs) for direct and indirect regeneration. The direct organogenesis was established from nodes and internodes on MS medium supplemented with cytokinins and auxins. The indirect organogenesis via callus phase was obtained from leaf, nodes and internodes on MS medium supplemented with different concentrations of PGRs. The high frequency shoot organogenesis were achieved directly from nodal explants were cultured on MS medium supplemented with 3.0 mg/L BAP+0.5 mg/L KIN +1.0 mg/L NAA. Indirect organogenesis callogenic frequency was optimized at the concentration of MS medium containing 1.0 mg/L BAP + 5.0 mg/L IAA. The callus was obtained from all the explants were used, among these explants internodal explants gave best result on MS medium supplemented with different concentrations of cytokinins and auxins for indirect organogenesis experiment. Indirect organogenesis the highest number of shoot regeneration was obtained in MS Basal Medium with 4.0 mg/L BAP + 0.5 mg/L KIN + 2.0 mg/L NAA from internodal explants. For root formation the regenerative shoots which were sub cultured on MS medium containing different ratios of auxins. The rooted plantlets were transferred successfully to the pots containing sterilized

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soil and were successfully hardened at greenhouse condition for 20 days then exposed to the natural environment. This is the first successful micropropagation report of an efficient and rapid in-vitro clonal propagation protocol for C. alata by direct and indirect shoot organogenesis through various explants, which can be employed for conservation of this important medicinal tree species as well as the utilization of an biologically important active biomolecules. This protocol can be very useful to obtain plants from various explants, without the requirement of meristematic regions, enabling the obtainment of a higher number of plants in short period.

Keywords: Crescentia alata; medicinal plant; plant growth regulators; Callus induction; shoot regeneration.

1. INTRODUCTION

Crescentia alata are commonly known as the Mexican Calabash tree which belongs to the family bignoniaceae is a flowering plant and it is an ancient tree of American tropics [1]. This is an important medicinal tree which is native to Southern Mexico and Central America south to Coast Rica and has been naturalized in India and other parts of the world for its novelty of potential medicinal uses [2]. It is an evergreen tree reaching 6 to 10 m in height with a broad, irregular crown composed of long, spreading branches clothed in 5 to 15 cm long bright green leaves. The tree is most important ornamental in the landscape for its year round production of flowers and fruit, both of which are unusual. They have 5 cm wide flowers, which bloom at night, are yellow/green with red or purple veins, cup-shaped, and appear to emerge directly from the branches. These are followed by the emergence of the large, round and cannonball like fruit 7-10 cm diameter that are difficult to break into, with a smooth, hard shell, which hang directly beneath the branches. The fruits develop after pollination by bats and have a hard green woody shell. Inside there is a pulp that has medicinal applications. The tree grows in clayey soils with deficient drainage subject to frequent floods [3]. The flowering and fruiting season of this plant is from June to October [4].

The various part of the plant species is most frequently used for the effective treatment of diseases like Bronchitis, Whooping Cough, Asthma and those related to illnesses caused by the cold. In traditional medicine the leaves and fruit of calabash are a part of the herbal mixtures reported in various medicines for respiratory ailments, bronchitis, cough, colds, toothaches, headaches, as laxative, anti-inflammatory, vermifuge and febrifuge [5]. Virtually, all parts of the tree have been found to be useful. The woods from the trees which are used for tool handles, ribs in boat building, cattle yokes, the gourd for cups, containers and musical instruments [6]. It is strong, flexible, moderately hard, and heavy. It is used for firewood and construction in rural areas and in the manufacture of handles for agricultural implements [7]. The medicinal value of *C. alata* indicates that the use of various parts of this tree species as plant extracts may be helpful in overcoming the disorders/disease predominant in the many rural areas of the country.

Micropropagation via indirect regeneration can be ensured the quality and rapidly production of plantlets [8]. Meanwhile, the response of various PGRs change during plant tissue culture can be varied from species to species and also this response depends on the ability of tissues to response and the types of PGRs [9]. The potential of two different PGRs auxins and cytokinins play an important role in direct and indirect regeneration in the micropropagation such as callus induction and shoots development [10]. In recent years, *in-vitro* approaches have been used as an efficient tool for large-scale propagation of trees in short time [11]. Mohsen Hesami et al., indicated that the highest regeneration frequency (96.66%) as well as shoot number (3.56) were observed in MS medium supplemented with 1.5 mg/L BAP and 0.15 mg/L IBA of *Ficus religiosa* through seedling derived petiole segments via indirect organogenesis [12]. Furthermore, Jaydip and Undurthy reported that the highest shoot regeneration response and the maximum shoots per callus were achieved from petiole explants on MS medium containing 0.25 mg/L TDZ and 0.25 mg/L NAA from *Adhatoda vasica* Nees through Indirect shoot organogenesis from leaf explants [13]. Also, Susmita and Yogendra demonstrated that the Maximum shoots (4.14 ± 0.25) in direct organogenesis were obtained on BAP (2 mg/L) + KIN (1 mg/L) + AgNO3 (1 mg/L). The maximum indirect shoot multiplication (26.83 ± 2.38) was obtained on BAP (1 mg/L) + AgNO3 (2 mg/L) in the subculture passage [14]. There is

no report according to PGRs ratios in *in-vitro* regeneration via direct and indirect organogenesis in *C. alata*.

Bianca et al., [15] derived the effect of different hormones on multiple shoot induction of *Zeyheria montana* using the nodal segments. Their results showed that multiple shoots were induced on Woody Plant media supplemented with 0.1 mg·L−1 of thidi-azuron (TDZ) was best medium for maximum proliferation of shoots. Further, the 20 shoots rooted on half strength WP (Woody Plant) media containing 1.0 mg·L−1 of indole- 3-butyric acid (IBA). The plants were hardened to ex-vitro condition was done at a 70% success rate using different substrates. It was possible to store *Z. montana's* elite germplasm using in vitro cultures of media containing 2% sucrose plus 4% sorbitol for six months without subcultures.

Conventionally the *C. alata* species is propagated either by seed or by cuttings. In vegetative propagation success rate is always very low, and the time consumption is an important factor in vegetative propagation. *C. alata* became threatened species with the possibility of natural climatic change, low germination of seeds, habitat loss and suffered an extremely limited ability to migrate. Due to over exploitation and misuse of medicinal plants, we are facing the problem of losing our precious plant resource for future. Furthermore, rising demand with shrinking habitats may leads to the local extinction of many wild medicinal plant species. Different techniques for conservation of plants have been practiced worldwide, the most important being tissue culture as it produces multiple clones of a plant species with in limited time space and enhancement of biochemical status without disturbing the wild plant. This situation calls for effective and in time conservation measures to enrich our lives with the services of plants. Hence, there is an urgent need to develop alternative propagation techniques to fulfill the current requirement and also to conserve this plant species.

Considering the importance of this medicinal tree for medicinal uses, pharmaceuticals and industries, it can be exploited at the commercial level. Hence keeping the importance of this valuable medicinal tree in view, it was selected for present investigation. Some *In vivo* studies have been carried on *C. alata* but no research has been published on tissue culture aspects. We have already demonstrated the results on *in-vitro* callogenesis and regeneration from *in vivo* and *in-vitro* grown vegetative explants of *C. alata*. However, *In-vitro* regeneration via direct and indirect organogenesis from *C. alata* has not been reported so far. This is a simple, rapid, economical, high frequency regeneration protocol from various explants of *C. alata*, for large scale propagation. The large-scale production of *C. alata* through the *in-vitro* propagation attributes are the quality, cost effectiveness, conserve and maintenance of genetic diversity and long-term storage. Moreover the micropropagation technique may be utilized, in basic and advanced research in production of various biologically and economically important secondary metabolites, virus free plants, cryopreservation of elite woody species, applications in tree breeding and reforestation.

The main objective of this study is to investigate the selection of suitable explants and PGRs on *in-vitro* regeneration via direct and indirect organogenesis in *C. alata*. Thus the present study was undertaken to develop an appropriate, reproducible and efficient micro propagation protocol from *Crescentia alata* tree explants. This study focused on standardizing efficient techniques for callus induction and micropropagation with specific objectives of (i) Callus development from various explants of *Crescentia alata* (ii) Direct and Indirect organogenesis of *Crescentia alata* using different concentrations and combinations of plant growth regulators.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Material

The plant materials were collected from Botanical garden of Department of Botany, JJ College of Arts and Science, Pudukkottai, Tamilnadu. It was collected during the monsoon season. Young nodal stem plant (soft, non woody and green in color) and leaf explants (soft and green in color) were selected as an explants source. The identification and authentication of plant material was by the authorities of the Botanical Survey of India (BSI), Southern Regional Centre, Coimbatore and the specimen samples are deposited in the BSI (Ref no: BSI/SRC/5/23/2017/Tech/3525).

2.2 Media Preparation

The potential of two different PGRs auxins (1.0-5.0 mg/L IBA, IAA and IBA) and cytokinins (1.0-

5.0 mg/L KIN and BAP) were analyzed for the induction of the callus and shoot regeneration. The basal nutrient media MS (Murashige and Skoog) supplemented with different combinations of PGRs (auxin and cytokinin) were used [16]. All the chemicals and PGRs were used are analytical grade and purchased from Hi Media Pvt. Ltd., Mumbai, India.

For direct shoot regeneration Leaf, Node and Internodal explants were inoculated on MS medium supplemented with BAP (6-benzyl aminopurine), KIN (6-Furturyl aminopurine) and NAA (1-Naphthaleneacetic acid) in different combination with different concentrations. For indirect shoot regeneration Leaf, Node and Internodal explants were inoculated on MS medium supplemented with BAP, KIN (6-Furturyl aminopurine), IAA (Indole-3-Acetic Acid) and NAA (1-Naphthaleneacetic acid) in different combination with different concentrations. For indirect shoot regeneration a piece of callus (2-3 mm^2) was used as an explant. Callogenic frequency was optimized at the concentration of MS medium containing 1.0 mg/L BAP + 5.0 mg/L IAA. The *in-vitro* regenerated shoots obtained from calli were studied up to three subculture passages at 5-week intervals.

2.3 Explant Preparation and Culture Conditions

The freshly harvested young stem and leaves of *C. alata* were cleaned and dead and decaying parts are separated. The separated parts were washed with the running tap water for 30 minutes and then rinsed in a solution containing the surfactant Tween-20 (2 drops in 100 ml solution) to remove the dust particles from surface. Subsequently, they were surface sterilized with 0.1% (w/v) HgCl2 solution for 1-2 min and 70% ethanol followed by three to five rinses with sterile distilled water in a clean air cabinet (under laminar air flow.). The surface-sterilized explants were aseptically cut into 1-1.5 cm segments and were carefully inoculated onto the MS culture media.

2.4 Inoculation of Ex-plants

Stem and leaf segments about 1-1.5 cm. were prepared aseptically and were implanted vertically on MS medium supplemented with various concentrations of auxins and cytokinins, singly or in combination, for callus induction and shoot regeneration. The culture media consisted of MS salts augmented with 3% sucrose and gelled with 0.8% agar including different concentration of PGRs (Hi-Media, India). The pH of the media was adjusted with 0.1(N) NaOH or 0.1(N) HCL solutions prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 121°C and 15 psi for 20-25 min. The cultures were incubated on culture rack at 25-28°C under constant temperature. The culture rooms have uniform forced-air ventilation and a humidity range of 20-98% controllable to ±3%. For each experiment a minimum of 10 replicates were taken and experiments were repeated thrice. The test tubes containing culture media of different concentrations of PGRs were put into laminar air flow for 1 hour. All the cultures from different explants were maintained in controlled environment of growth room under illumination, provided by white fluorescent tube lights with a photoperiod of 16 hours. The cultures were visited everyday to observe their response to tissue culture, and data were recorded.

2.5 Rooting of *In-vitro* Regenerated Plantlets

The regenerated shoots (4-6 cm in length) were rooted on MS medium supplemented with different concentrations of IBA (Indole 3-butyric acid). The data for percentage of root formation, number of roots and root length per shoot was recorded periodically after 4 weeks of culture.

2.6 Hardening and Acclimatization

The shoots derived via direct and indirect organogenesis with well developed roots were treated in 1% bavisitin fungicide solution for 1 min and then rinsed under tap water and transferred to small cups filled with sterilized garden soil, sand and vermiculite (1:1:1) for hardening. These cups were covered with transparent polythene cover. The polythene covers were perforated with small holes to maintain humidity. After 2 weeks the polythene covers were removed from cups. After 2 months, the plants were transferred to garden soil in earthen pots and kept under sunlight, initially for a short time and gradually the time was increased. After 3-4 months the plants were transferred to field.

2.7 Statistical Analysis

For all the experiments were conducted in completely randomized design and were repeated twice with 30 replicates per treatment.

Analyses of comparisons of means were performed using the Dunnett multiple comparison test.

3. RESULTS

In the present study, direct shoot organogenesis was achieved from nodal explants where as indirect shoot organogenesis was achieved from via callus phase from internodal explants of *C. alata* in MS basal medium. Among the different PGRs tested the maximum Shoot regeneration and number of shoots depended upon the type of PGRs and its concentrations were used.

3.1 Direct Organogenesis-induction of Organogenesis Directly from the Explants

To achieve direct regeneration leaf, nodal and internodal explants were cultured on MS medium supplemented with different concentrations and combinations of plant growth hormones. The experiment was repeated 3 times (Table 1). The proliferation of shoots achieved directly from nodal explants and shoots were formed maximum (3-5) with early response and Maximum frequency of shoot initiation and shoot multiplication (4.8 ± 0.83) was observed on MS medium supplemented with 3.0 mg/L BAP + 0.5 mg/L KIN +1.0 mg/L NAA respectively. The shoots were initiated better with green and healthy leaves within 10 - 12 days from nodal explants. For direct shoot proliferation from the nodal explant could be adequate amount of cytokinin along with lower concentration of auxins supplied in MS medium. However, an increase or decrease in the concentrations and combinations of 2,4-Dichlorophenoxyacetic acid and KIN showed a negative trend in shoot proliferation. As many as three or five shoots per nodal explant were obtained with a combination of BAP (3.0 mg/L), KIN (0.5 mg/L) and NAA (1.0 mg/L), in 25 to 35 days of incubation although shoot production was associated with callus formation (Fig. 1). The morphogenic responses of nodal explants cultured on MS medium with different PGRs are presented in Table 1.

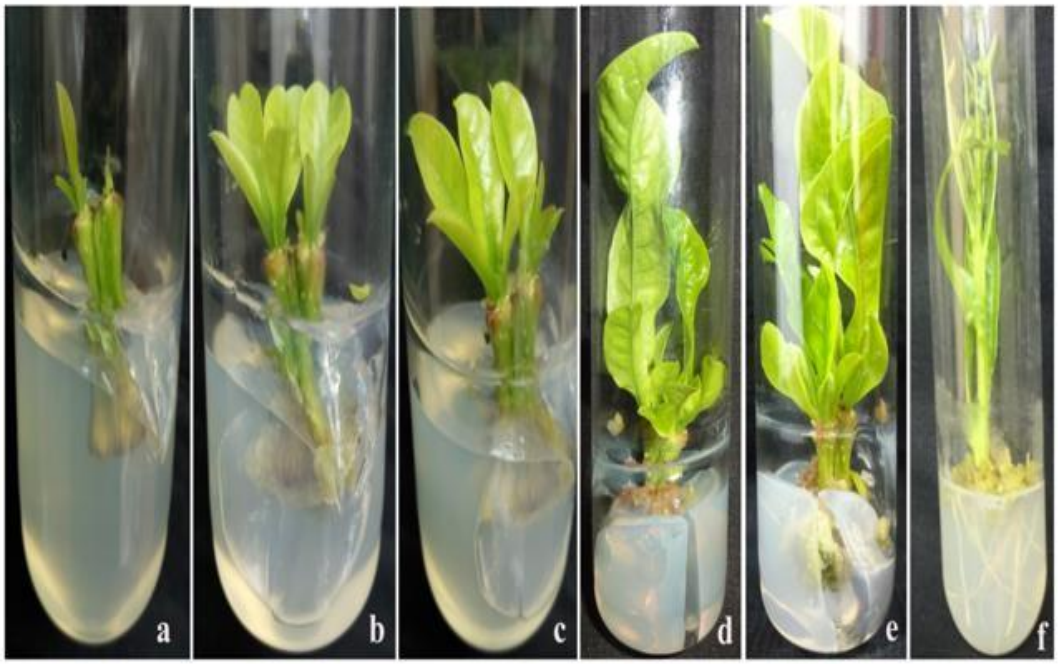

Fig. 1. Direct regeneration from Nodal explants of C. alata (a-f). a. Nodal Explant on regeneration medium with Shoot initiation. b & c. Shoot induction and proliferation from cotyledonary node on MS full strength basal medium supplemented with 3.0 mg/L BAP + 0.5 mg/L KIN + 1.0 mg/L NAA after 10-12 days of culture. d & e. Shoot elongation after 25-35 days in culture on MS full strength basal medium supplemented with 3.0 mg/L BAP + 0.5 mg/L KIN + 1.0 mg/L NAA. f. Formation of roots from the regenerated shoots subcultured on MS half strength basal medium with BAP 2.0 mg/L IBA + 1.0 mg/L NAA within 35 days

Table 1. Effect of cytokinins and auxins on direct shoot organogenesis from nodal explants of *C. alata*

S.No	MS+3%Sucrose+(mg/L) PGRs	No of explants cultured	Percentage of response (%)	Days of response	Number of Shoots (Mean±SD)
1	1.0 BAP + 0.5 KIN + 1.0 NAA	100	75	36	3.6 ± 0.53
2	2.0 BAP + 0.5 KIN + 1.0 NAA	100	78	36	3.8 ± 0.83
3	3.0 BAP + 0.5 KIN + 1.0 NAA	100	79	36	4.8 ± 0.83
4	4.0 BAP + 0.5 KIN + 1.0 NAA	100	72	36	3.2 ± 0.83
5	5.0 BAP + 0.5 KIN + 1.0 NAA	100	75	36	3.8 ± 1.03

3.2 Indirect Organogenesis-initiation of the Callus from Leaf, Nodal and Internodal Explants

For callus initiation the leaf, nodal and internodal explants were transferred to MS medium supplemented with various concentrations and combinations of plant growth hormones. The experiment was repeated thrice. Within 12-15 days of inoculation, rapidly proliferating callus induction was obtained from the cut surface of all the explants were cultured on MS medium supplemented with various PGRs. The maximum amount of callus induction was observed from MS medium supplemented with 1.0 mg/L BAP + 5.0 mg/L IAA from intermodal explants (Table II). The color of the callus was yellowish green which indicates the plant cells undergo stress when inoculated on MS nutrient media in culture condition. All other combinations of plant growth hormones were capable of producing more or less poor result and required more time for initiation, and proliferation was not rapid. Both auxins and cytokinins are major growth regulators that have profound influence on various phenomenons of cell division, callus induction and regeneration. Stem explants showed good proliferation of the callus from all over the surface of the explants in 15 days when the concentration of NAA was increased to 5.0 mg/L (Table 2).

3.2.1 Regeneration from the callus

Callus was obtained from stem explants when cultured on MS medium supplemented with 1.0 mg/L BAP + 5.0 mg/L IAA. These callus pieces of approximately 0.5 to 1 cm2 were incubated on MS medium supplemented with various combinations of plant growth hormones in replicates of 10 each and the experiment was repeated three times. The maximum number of shoots was obtained from a combination of 4.0 mg/L BAP + 0.5 mg/L KIN + 2.0 mg/L NAA from internodal explants (Table 3, Fig. 2).

Table 2. Effect of PGRs on callus induction and proliferation from intermodal explants of *C. alata*

S.No	MS+3% Sucrose + (mg/L) PGRs	Color and Nature of Callus	Days of Respons e	No of explants cultured	Percentage of response (%)	Frequency of Callus formation
Control	Ms + Without PGR	No Callusing	-	-	-	-
1	1.0 BAP + 1.0 IAA	Yellowish green in color, fast Growing and compact	28	100	85	+
2	1.0 BAP + 2.0 IAA		28	100	88	+
3	1.0 BAP + 3.0 IAA		28	100	93	++
4	1.0 BAP + 4.0 IAA		28	100	92	++
5	1.0 BAP + 5.0 IAA		28	100	95	+++

+++ Good response; ++ Medium response; + Low response; - No response

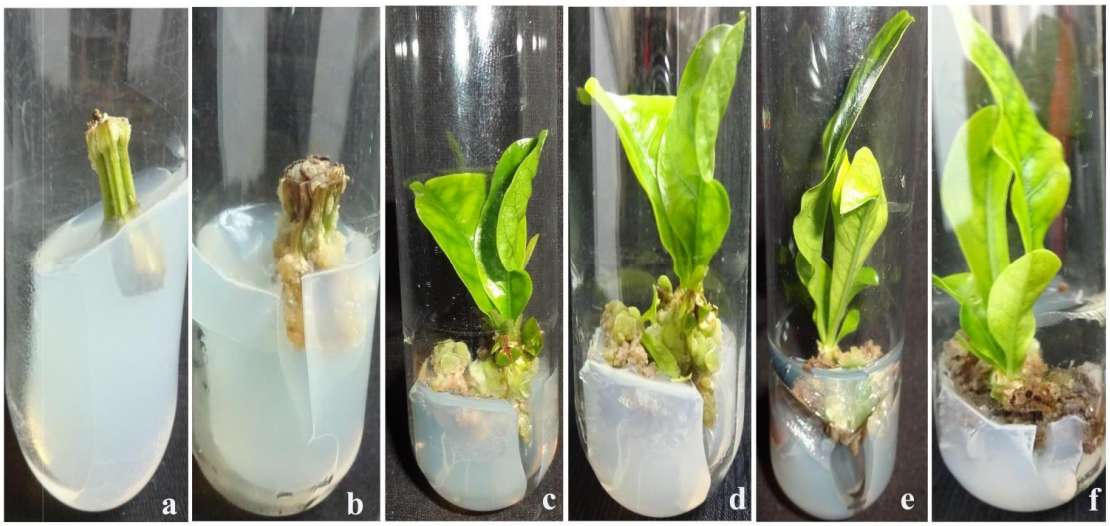

Fig. 2. Indirect regeneration from internodal explants of *C. alata* (a-f). a. Intermodal explants cultured on callus induction medium MS full strength basal medium + 1.0 mg/L BAP + 5.0 mg/L IAA at 0 days. b. Intermodal explants showing expansion and callus initiation after 12-15 days of inoculation. c & d. Shoot induction and development from subcultured intermodal derived callus after 21 days of culture on MS full strength basal medium + 4.0 mg/L BAP + 0.5 mg/L KIN + 2.0 mg/L NAA. (Callus subcultured for five weeks). e & f. Shoot elongation after 25-35 days in culture on MS full strength basal medium + 4.0 mg/L BAP + 0.5 mg/L KIN + 2.0 mg/L NAA

Table 3. Influence of various PGRs on indirect shoot organogenesis from intermodal derived calli of *C. alata*

S.No	MS+3% Sucrose + (mg/L) PGRs	No of explants cultured	Percentage of response (%)	Days of response	Number of Shoots (Mean ± SD)
1	1.0 BAP + 0.5 KIN + 2.0NAA	100	72	36	3.2 ± 0.83
2	2.0 BAP + 0.5 KIN + 2.0NAA	100	74	36	3.6 ± 0.53
3	3.0 BAP + 0.5 KIN + 2.0NAA	100	75	36	3.8 ± 1.03
4	4.0 BAP + 0.5 KIN + 2.0NAA	100	78	36	4.6 ± 0.54
5	5.0 BAP + 0.5KIN + 2.0NAA	100	75	36	3.8 ± 0.83

Table 4. Effect of IBA on *in-vitro* rooting in microshoots of *C. alata* on half strength MS-medium

S.No	Half strength MS+3% Sucrose + (mg/L) PGRs	Number of explants cultured	Percentage of response (%)	Root lengths in cm (Mean ± SD)	Mean root numbers Mean ± SD	Days of rooting in weeks
1	0.5 IBA + 1.0 NAA	100	53	2.5 ± 0.4	5.5±0.6	5^{th} week
2	1.0 IBA + 1.0 NAA	100	67	3.5 ± 0.5	6.1±0.5	
3	1.5 IBA + 1.0 NAA	100	72	3.7 ± 0.5	6.8±0.5	
4	2.0 IBA + 1.0 NAA	100	78	4.8 ± 0.5	7.5±0.3	
5	2.5 IBA + 1.0 NAA	100	71	3.6 ± 0.5	6.5±0.5	

3.3 Rooting of the *In-vitro* Formed Shoots

The regenerated shoots (4-6 cm in length) were rooted on MS medium supplemented with 3% sucrose and different concentrations of IBA (Indole 3-butyric acid). Different media compositions were tried among which the best response for rooting was observed in half strength MS basal supplemented with 2.0 IBA + 1.0 NAA. Maximum frequency of roots (78%), root number (7.5 ± 0.3) and root length (4.8 ± 0.5) was observed on 2.0 mg/L IBA + 1.0 NAA within 35 days of culture without the intervening basal callus (Table 4). On the other hand, full strength MS basal medium did not show favored rooting even when tried with the same concentration of IBA. The data for percentage of root formation, number of roots and root length per shoot was recorded periodically after 4 th weeks of culture. The PGRs IBA is considered to be a potential auxin that induces rooting of *in-vitro* regenerated shoots of several tree species *Jatropha curcas* [17], *Albizia odoratissima* [18].

3.4 Transferring the *In-vitro* Raised Plants to the Greenhouse

The shoots derived via direct and indirect organogenesis with well rooted *in-vitro* plants were treated in 1% bavisitin fungicide solution for 1 min and then rinsed under tap water and transferred to small cups filled with sterilized garden soil, sand and vermiculite (1:1:1) for hardening. These cups were covered with transparent polythene cover. The polythene covers were perforated with small holes to maintain humidity. After 2 weeks the polythene covers were removed from cups. After 1 month, the plants were transferred to garden soil in earthen pots and kept under sunlight, initially for a short time and gradually the time was increased. Hardening was applied for two weeks in a greenhouse (80-85% relative humidity and 27 ± 2°C) but only 50-55% of the plants survived the period in the greenhouse. After 2 months the plants were transferred to field. 75-80% of the plantlets survived during acclimatization. The *in-vitro* regenerated plants did not show any detectable variation in morphological or growth characteristics compared to the parent plant. The *in-vitro* derived plantlets were similar to that of *in-vivo* plants.

4. DISCUSSION

In this study, direct and indirect regeneration systems of *C. alata* were successfully developed. According to these results, clone differences were observed in both regeneration types, i.e. direct and indirect regeneration. Although callus induction has been achieved with many species in bignoniaceae members *Oroxylum indicum* [19], Hardwood tree species [20], *Tecomella undulata* [21], *Catalpa bungei* [22], *zamioculcas zamiifolia* [23]. So far there has been no report on callus cultures or clonal multiplication of *C. alata*. Direct and indirect Shoot regeneration from callus culture and nodal explants of *C. alata* was achieved.

The effects of PGRs, basal media strength and sucrose were studied using different concentrations (1.0 to 5.0 mg/L) of indole-3-acetic acid (IAA), indole- 3-butyric acid (IBA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy-acetic acid (2,4-D) alone or in combination with (1.0 to 5.0 mg/L) of 6-benzylamino purine (BAP) and 6-Furturyl aminopurine (KIN). The results showed that NAA in combination with BAP gave a higher callusing percentage (80 to 100%) when compared to the other PGRs at the same concentrations. When an auxin was supplied in combination with BAP, a significant increase in callusing percentage or degree of callusing was observed.

For callus induction and shoot regeneration are dependent on two factors i.e. PGRs concentration and explants types. The explants type was an important factor affecting the formation of callus and regeneration from the callus, direct regeneration from the explants depended on the choice of explants i.e. nodal segments could produce more shoots per explants than intermodal explants. However, the percentage response of callus formation was significantly increased when IAA was added to the medium in combination with BAP (Table 2). In our experiment MS medium was found to be ideal for the callus cultures. For the induction of callus cultures, we preferred ms medium containing lower concentrations of cytokinin and higher concentration of auxin to be the best combination. Our experiments showed NAA with BAP to be essential for callus induction and growth, in agreement with previous reports on *Solanum dubium* [24] *Carica papaya* [25].

4.1 Direct Organogenesis

The direct shoot organogenesis in *C. alata* depended on the type of explant used. Of the different explants used in this study, direct shoot regeneration was obtained only from nodal

explants, while the petiole and internodes failed to initiate shoot buds. Shoot bud proliferation from the nodal explants required a low amount of auxin to BAP and KIN combination. Comparatively, the direct shoot induction response as well as the shoot number (4.8 ± 0.83) was higher than the callus mediated cultures. Of the various treatments MS medium supplemented with BAP (3.0 mg/L), KIN (0.5 mg/L) and NAA (1.0 mg/L) proved to be best for direct shoot bud induction and proliferation in *C. alata*. Earlier, NAA along with BA and KIN produced highest direct organogenesis from hypocotyl explant of *Aegle marmelos Correa* [26]. For direct organogenesis the shoot regeneration from cotyledonary node explants has been achieved in many tree species such as *Dalbergia sissoo* [27], *Cleistanthus collinus* [28], *Terminalia bellirica* [29], *Balanites aegyptiaca* [30], *Bambusa glaucescens* [31].

4.2 Indirect Organogenesis

For Indirect organogenesis the highest frequency of regenerating callus response and the maxium number of shoots per callus explants (4.6 ± 0.54 cm) with maximum shoot length were achieved on MS medium supplemented with 4.0 mg/L BAP + 0.5 mg/L KIN +2.0 mg/L NAA from intermodal explants (Fig. 2). These findings are in accordance with the previous report on *Abutilon ranadei* where combination of KIN and NAA proved superior for induction of shoots from organogenic callus [32]. It was previously reported that low concentration of an auxin in combination with a cytokinin positively modifies the shoot induction frequency and their growth [33,34]. We also found that addition of 0.5 mg/L kinetin with BAP enhances shoot formation. However, the shoot regeneration has completely failed in *Tinospora* species, *Tinospora cordifolia* [35,36] *Tinospora formanii* [37].

The *in-vitro* rooting responses in microshoots of *C. alata* are presented in Table 4. Auxins are involved in the process of adventitious root formation, but these hormones affect in-vitro rooting of various species differently [38,39]. The results showed that the addition of auxin IBA or NAA effectively could promote induction of roots. Well developed shoots from the culture were excised and transferred to rooting medium (half strength MS) augmented with IBA or NAA. The results showed that the addition of auxin IBA or NAA could effectively promote induction of roots. The highest rooting frequency (78%) was achieved when *in-vitro* regenerated shoots were transferred to half strength MS medium supplemented with IBA (2.0 mg/L) with NAA (1.0 mg/L) (Table 4 and Fig. 1f). However in our experiments we achieved good root formation with adding IBA hormones but with a reduction of salt concentration in the medium.

5. CONCLUSION

In conclusion, thus the findings of the present investigation helped to find out the accession with medicinal qualities and to use for the mass propagation of medicinally important tree species through *in vitro* regeneration. *Crescentia alata* have great potential use as phytomedicine as they have antimicrobial and anticancer activities. An efficient and reproducible method for Direct and Indirect shoot regeneration protocols were developed for large-scale multiplication, propagation and conservation of *C. alata* using various explants. Considering the medicinal and economic importance of the plant, this current protocol offers a potential system for rapid multiplication as well as germplasm conservation. This is the first report on *in-vitro* propagation through Direct and Indirect shoot organogenesis of *C. alata*. This protocol will be helpful for conservation of other *Crescentia* species as well as medicinally important tree species.

CONSENT

It's not applicable.

ETHICAL APPROVAL

It's not applicable.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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