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Research article

In vitro response of reproductive organs of *Crescentia alata* kunth to callogenesis, an important multipurpose medicinal tree

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Key words: *Crescentia alata,* Bignoniaceae, *In vitro* callogenesis, reproductive explants, ovary culture, plant growth regulators.

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Abstract

Crescentia alata is an important species in the trumpet-flower family. It is an important medicinal and commercially valuable tree which belongs to the family Bignoniaceae and it is commonly called as Mexican Calabash tree. *C. alata* is a part of the herbal mixtures reported in various traditional medicines for respiratory ailments, bronchitis, cough, colds, and toothaches, headaches, as laxative, anti-inflammatory and febrifuge. Plant tissue culture is a fast and efficient tool for developing new varieties in a comparatively short time. Various explants excised from reproductive organs of *Crescentia alata* were inoculated on Murashige and Skoog (MS) medium. It was supplemented with different concentrations and different combinations of PGRs (BAP, KIN, IBA, NAA, IAA, 2, 4-D) for callogenesis. Initial callus induction was found to be highest in style with ovary explants on 2, 4-D 3.0 mg/l and Kinetin 0.5 mg/l in 13th day of the culture, respectively. Style with ovary found to be the best explant for callus induction and growth (80%). Use of Style with ovary explant source is the best among various reproductive parts already explored. 2, 4-D & Kinetin seems to be better growth hormone for both callus induction and callus growth. This protocol can be helpful to propagate male and female plants swiftly by subsequent embryogenesis and organogenesis.

Introduction

Crescentia alata belonging to the family Bignoniaceae and it is a flowering plant that is native to Southern Mexico and Central America and it is naturalized in India [1]. In India, it is found in Delhi, Uttar Pradesh, Bihar, West Bengal, Sikkim, Assam, Tripura, Gujarat, Maharashtra and Tamil Nadu. It grows at elevations from sea level to 800m, in areas with an average annual precipitation between 1,500 to 1,300mm and an average annual temperature of 26°C. It is an evergreen tree reaching 6 to 10m in height with a broad, irregular crown composed of long, spreading branches clothed in 5 to 15cm 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 [2].

The flowering and fruiting season of this plant is from June to October [3]. The flowers of the calabash tree are an example of its many unique attributes. Calabash flower undergo pollination and set fruit within a period of 2-3 months at the beginning of the rainy season. The flower buds of the calabash tree grow directly on the woody bark of the tree. The buds grow directly on the trunk and large branches of the tree because only these sturdier parts of the tree can hold the weight of the very heavy mature fruits. *Crescentia* flowers possess a fused corolla (collective term for flower petals) that is generally tubular in shape. Once the floral tube starts to emerge from the bud, it only takes a matter of days for the flower to grow to its full size. The outside of the petals is a deep red, while the inside is cream colored. Once mature the morrow flower only fully opens at night. *C.alata* trees blooms at night to accommodate their pollinators, nectivorous bats. *Crescentia alata* flowers are precisely the right size and shape to accommodate the face of the bats. The stamens are located at the entrance of the floral tube where they are deposit pollen onto furry bat faces as they reach to the very back of the flower to feed on nectar. The floral tube itself is durable in texture, with a slightly rubbery feel. The flowers of calabash close during the day to protect against loosing water to the intense heat of their natural habitat. The pollination once occurred and the floral tube fallen to the ground, the newly fertilized and developing fruit can be seen nestled

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in between the floral brackets that previously enclosed the immature flower just weeks before. Over the next several months the fruits will greatly increase in size and weight to produce a bizarre, but very useful, fruit [4].

The plant is used most frequently for the effective treatment of diseases like Bronchitis, Whooping Cough, Asthma and those related to illnesses caused by the cold. Good results have been seen in the treatment of otalgia (earaches). In traditional medicine Vermifuge properties are attributed to it. The fruit of *C. alata* is a part of the herbal mixtures reported in various traditional medicine for respiratory ailments, bronchitis, cough, colds, toothaches, headaches, as laxative, anti-inflammatory and febrifuge [5]. Virtually, all parts of the tree have been found to be useful. The woods from the trees of *C. alata* which are used for tool handles, ribs in boat building, cattle yokes, the gourd for cups, containers and musical instruments. The fruit is reported to have medicinal application [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 ability to produce haploid plants is a tremendous asset in plant breeding and genetic studies. Heritability studies are simplified, because with only one set of chromosomes, recessive mutations are easily identified by genetic engineering methods [8]. The haploid plants were first discovered in *Datura stramonium* by A.D Bergner in 1921 [9]. After the initial reports of the successful production of haploids from another culture in *Datura* species through tissue culture technology, many haploid species have been obtained in more than 150 species belonging to 23 families of angiosperms [10], while Production of haploid plants through gynogenesis by culturing unfertilized ovaries was first described in barley [11]. *In vitro* gynogenesis involves the culture of unfertilized ovules or ovaries while Androgenesis is the process by which haploid plants develop from the male gametophyte. The Gynogenetic haploids may develop directly or indirectly via tissue culture from the callus as in androgenesis. Ovary and flower bud cultures have generally been found to be more efficient than ovule culture because of the less intrusive manipulation. Plant breeding can produce lines that are more resistant and have higher yield; however standard plant breeding takes many years. Doubled haploid lines can be produced in one step by tissue culture of flower parts. Gamete cells may be manipulated to produce embryos, in contrast to normal fertilization of ovules by pollen grains. Induced or spontaneous chromosome doubling can generate completely homozygous doubled haploid plants [12].

The gynogenenic experiments are usually carried out using ovaries or unfertilized ovules, although entire immature flower buds. The ovaries are easier to dissect than ovules without damaging the female gametophyte. Direct gynogenesis usually involves the egg cell, synergids or antipodals with organized cell divisions leading first to the formation of proembryos and then to well-differentiated embryos. In indirect gynogenesis, callus may be formed directly from the egg cell, synergids, polar nuclei, and antipodals or may develop from proembryos. Plants regenerated from callus maybe haploid, diploid or mixoploid.

The commonly used media for another culture include MS (Murashige and Skoog, 1962), N6 or variations on these media N6 [13]. Complex organic compounds, such as potato extract, coconut milk and casein hydrolysate, have been added to the culture media for better results. Various cultural conditions, such as temperature and light may also affect androgenic response. The cultures were incubated on culture rack at 25-28°C under constant temperature. The culture room has uniform forced-air ventilation and a humidity range of 20-98% controllable to ± 3%.

The study was early done to evaluate various reproductive explants and efficacy of growth regulators for callogenesis on MS media (Murashige & Skoog, 1962) supplemented with certain growth regulators. We have already demonstrated the results on callogenesis from *in vivo* and *in vitro* grown vegetative explants of *crescentia alata*. This paper, however, includes the development of protocol for callogenesis from field grown reproductive organs of *C. alata*. The study might be promising for commercial propagation of male and female plants. This protocol may help to improve the understanding in developing synthetic seeds and transgenic plants in *crescentia alata*. The purpose of this work was to study the effect of different plant growth regulators on *in vitro* callus induction and growth of floral explants in *C. alata*.

Experimental

Materials and methods

Collection and authentification of plant material

Unopened floral buds were taken from trees growing at Botanical garden of Department of Botany, JJ Arts and Science College, Pudukkottai, Tamilnadu (figure1-a&b). The identification and authentification 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).

Media preparation

The potential of two different PGRs auxins (1.0-5.0 mg/l 2, 4-D, IAA or IBA) and cytokinins (1.0-5.0 mg/l KN or BAP) were analyzed for the induction of callus. The basal nutrient media MS (Murashige and Skoog, 1962) supplemented with different concentrations and

combinations of PGRs (auxin and cytokinin) were used [14].

Explant surface sterilization

Explant types employed were petal, sepal, stigma, stigma plus ovary, ovary and floral bud. Explants were surface sterilized by submerging in 70% ethanol plus one to two drops of Tween 20 (surfactant) for one minute and in 0.5% NaOCl for two to three minutes under laminar air flow.

Inoculation of ex-plants

The buds were dissected in sterile Petri dish with the help of forcep and scalpel. Then, the explants were collected and cultured on MS (Murashige and Skoog, 1962) basal medium enriched with constant concentrations of 2,4-D (1.0 mg/l to 5.0 mg/l) alone and in combination with KIN (0.5 mg/l and 1.0 mg/l) and also in combination of 2,4-D 1-5mg/l + 1mg/l BAP + 0.5mg/l NAA and 2,4-D 1-5mg/l + 1mg/l KIN+ 2mg/l BAP for callus induction. Finally all the explants were prepared aseptically and were implanted vertically on MS medium prepared with different concentrations of auxins and cytokinins, singly or in combination for callus induction. The medium containing 3% sucrose was solidified with 8% agar (including different concentration of PGRs). The pH of the media was adjusted to 5.8 with 1N NaOH or 1N HCL solutions prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 121°C and 15 psi for 20-25min.

The cultures were kept in growth chamber at 25 ± 2°C and incubated under controlled humidity. 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 were maintained in controlled environment of growth room under illumination, provided by white fluorescent tube lights (2500 lux) with a photoperiod of 16hours. Callus initiated at the cut end of explants after 13 days of culturing. Cultures were visited regularly to observe their response to tissue culture and data were recorded.

Results and discussion

Differences among various treatments were observed when constant concentrations of 2,4-D (1.0 mg/l to 5.0 mg/l) alone and in combination with KIN (0.5 mg/l and 1.0 mg/l) and also in combination of 2,4-D 1-5mg/l + 1mg/l BAP + 0.5mg/l NAA and 2,4-D 1-5mg/l + 1mg/l KIN+ 2mg/l BAP were added to MS medium for the induction and proliferation of callus (Table 1). Callus was formed initially on the cut ends of the ovary explants on callus induction medium after 13 days of culture (Figure 1 d). Callus covered almost all the whole explant. The callus formation was strongly dependent on the combination and concentration of the growth regulators supplied to the culture media as well as on the genotype. After 4 weeks, soft and friable yellowish calli were produced on culture media. The best and maximum callus response was found with 2, 4-D 3mg/l + KIN 0.5 mg/l followed by either 2, 4-D 3mg/l + KIN 1mg/l + 2mg/l BAP and/or 2, 4-D 4mg/l + KIN 1mg/l + 2mg/l BAP with 80%, 60% and 50% respectively. The colour of callus produced was yellowish and whitish green. Approximately 80% callus was soft and friable, while 20% was compact. Of the various concentrations and different combinations of PGRs tested, MS medium supplemented with KIN and 2, 4-D were found to be ideal for callus induction and proliferation of tissue. The highest response of callus proliferation was observed on the treatments of 2, 4-D 3mg/l + KIN 0.5mg/l while the least response of callus proliferation was observed on the medium supplemented with 2,4-D 1-5mg/l + 1mg/l BAP + 0.5mg/l NAA (80%) (Table 1 and figure 1 e & f). Floral bud, stigma, anther, sepal, petal and filament explants obtained intermediary position for callus induction showing significantly less callus induction and some explants are unresponsive.

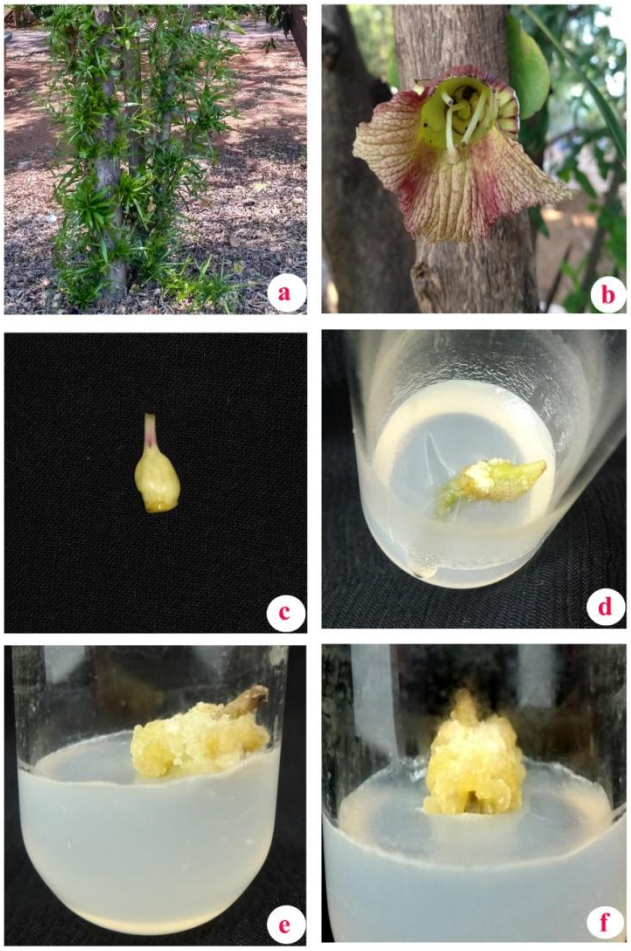


Figure 1. a- *In vitro* grown of *C. alata*, b- Flower, c- Style with ovary explants, d- Callus induction from ovary explants, e and f- After 35 days of inoculation.

Table 1. ***In vitro*** **responses of different Plant Growth Regulators (Cytokinin and Auxin) on Callus induction from ovary explant of** ***C. alata***

Explant type	Conc. of growth regulator (mg/L)				Frequency of Callus formation	Days of Response	Morphogenetic response	% of response
	2,4-D	KIN	BAP	NAA				
Style with ovary	0.0	0.0	0.0	0.0	-	-	-	-
	1.0	0.5	-	-	+		Yellow friable callus	30
	2.0	0.5	-	-	++		Yellow friable callus	50
	3.0	**0.5**	**-**	**-**	**+++**		**Yellow friable callus**	**80**
	4.0	0.5	-	-	++		Yellow friable callus	50
	5.0	0.5	-	-	++		Yellow friable callus	60
	1.0	-	1.0	0.5	+		White friable callus	30
	2.0	-	1.0	0.5	+		White friable callus	40
	3.0	-	1.0	0.5	+		White friable callus	30
	4.0	-	1.0	0.5	+	**35**	White friable callus	30
	5.0	-	1.0	0.5	+		White friable callus	30
	1.0	1.0	2.0	-	+		Yellow friable callus	40
	2.0	1.0	2.0	-	+		Yellow friable callus	40
	3.0	1.0	2.0	-	++		Yellow friable callus	60
	4.0	1.0	2.0	-	++		Yellow friable callus	50
	5.0	1.0	2.0	-	+		Yellow friable callus	40

'+' indicates the degree of response; '-' indicates no response
+++ Good response; ++ Medium response; + Low response

This is the first report on the induction of callogenesis from floral parts of *C. alata*. Plant hormones are among the most important physiological factors affecting the callus growth of plants *in vitro*. Several studies have been reported regarding the effects of plant growth regulators on callus growth of different plants. The major differences in the response of different plants and different explants to tissue culture conditions lie in the ratio of auxins to cytokinins Skoog and Miller (1957), [15]. A significant effect of the combination of PGRs 2,4-D and KIN on callus formation in *barringtonia racemosa*, leaf and endosperm explants culture was reported by Dhiya dalila *et al.,* (2005), [16]. The similar effect of 2,4-D and kinetin was also reported by Amiri *et al.,* (2011), [17], Namrata Shanu Gupta *et al.,* (2014), [18] and Talukdar and Ahmed (2004), [19]. Furthermore the percent response to callus induction in 2, 4-D and Kn combination was directly proportional with the increasing concentration of PGR showing a 89.7 % response in 2 mg L -1 2,4-D and 1.6 mg L-1 Kn followed by 1 mg L-1 2, 4-D callus induction from nodal explant in 2, 4-D and Kn combination is in agreement with the results, reported by Aryal and Joshi (2009) [20] where 2, 4-D and Kn combination was found to be effective in inducing callus from stem explants [20]. In contrast to the present findings, Moitreyee Saikia *et al.,* (2012) and obert *et al.,*(2005) reported the highest growth of the callus on MS medium supplemented with quite higher amount of growth hormones BAP and NAA within 30 - 45 days of culture [21] and [22]. In the current study, higher callus proliferation in ovaries suggests that these explants can be used to obtain an efficient propagation system, employing somatic embryogenesis.

Conclusion

2, 4-D & Kinetin seems to be better growth hormone for both callus induction and callus growth. The current experiment was an initial step towards the mass production of calli from different explants of flower. Overall, style with ovary explants emerged better for callogenesis among the reproductive explants employed. However, style with ovary initiated callus earlier than other explants were used for culture. These reproductive parts are pro-embryogenic in nature and might have more amounts of growth hormones there leading to an earlier and better response for callus induction. The ovarian tissues are an explants source could be suggested as the best among various reproductive parts for callogenesis. This protocol might be helpful to propagate male and female plants swiftly by subsequent embryogenesis and organogenosis. It could also be used in the study of embryo development, fruit development, and different aspects of fruit physiology including respiration, maturation and disease. Ovary culture has also been successful in inducing polyembryony. Poly-embryo may develop through tissue culture technology from the various parts of the ovary. These poly-embryos develop into many shoots instead of a single plantlet. Rare hybrids can also be produced on ovary culture and the physiology of fruit development can be studied by ovary culture. It is also helpful in developing synthetic seeds and transgenic plants of *Crescentia alata*.

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