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Research article

In vitro culture medium and explant type effect on callogenesis and shoot regeneration in *Crescentia alata* Kunth

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Key words: *Crescentia alata*, Bignoniaceae, *In vitro*, callogenesis, organogenesis, 6-benzylaminopurine.

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Abstract

The present investigation deals with the callus induction and shoot regeneration of Crescentia alata. C. alata belongs to the family Bignoniaceae and it is commonly called as Mexican Calabash tree. The destruction of this plant species is severe and planting of seedlings is almost negligible. Due to low germination and unsuccessful vegetative propagation, *in vitro* regeneration of *Crescentia alata* through micropropagation was studied as a tool for large scale propagation. In present investigation which is first report on *in vitro* propagation of *C. alata*. Different treatments were employed for surface sterilization of explants. Stem and leaf segments cultured on MS medium supplemented with different concentrations and different combinations of PGRs (BAP, IBA, NAA, IAA, Kn and 2, 4-D).The maximum amount of callus induction was observed from BAP 2mg/l + IBA 1 mg/l+ IAA 0.5mg/l respectively .The proliferation of shoots achieved directly from nodal explants and shoots formed was maximum (3-5) with early response on MS medium supplemented with BAP 3 mg/l + IBA 1 mg/l+ IAA 0.5mg/l respectively. In this study the regeneration system is efficient for callus induction and shoots development, showing high potential for advanced cellular and molecular studies. This study may contribute in conservation management and production of secondary metabolites of this plant species especially in India.

Introduction

Crescentia alata commonly known as the Mexican Calabash tree belonging to family Bignoniaceae 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. The tree is most outstanding in the landscape for its year-round production of flowers and fruit, both of which are unusual. The 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 fruit, 12 cm to 30 cm in diameter, with a smooth, hard shell, which hang directly beneath the branches. The fruit develop after pollination by bats and has 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 of this plant is from June to October [3].

The extract of the fruit is effective in the treatment of fever. 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 [4]. Virtually, all parts of the tree have been found to be useful. The wood is used for tool handles, ribs in boat building and cattle yokes; and the gourd for cups, containers and musical instruments. The fruit is reported to have medicinal application [5].

Crescentia alata is a seed propagating plant. The seeds, however, have short viability and are prone to microbial attacks. The tree bears fertile seeds in 15-20 years. Due to over exploitation and misuse of medicinal plants we are facing the problem of losing our precious plant resource for future. This situation calls for effective and in time conservation measures to enrich our lives with the services of plants. In this regard various research groups across the world have attempted conservation of plants to protect biodiversity [6,7].

Exploitation of medicinal plants for commercial use coupled with the destruction of underground parts, slow reproduction, slow growing and habitat-specific nature, are the crucial factors in meeting the goal of sustainability [8,9]. Considering the importance of this medicinal tree for medicinal uses, pharmaceuticals and industries, it can be exploited at the commercial level. The medicinal value

indicates that the use of various parts (leaves, roots, bark, etc.) of this tree species as plant extracts may be helpful in overcoming the disorders/disease predominant in the many rural areas of the country.

Production of callus and subsequent regeneration of plants are the main stages in the biotechnological manipulation *in vitro*. The compositions of the medium and culture conditions are one of the most important things that determine the effect of organogenesis [10]. Furthermore, rising demand with shrinking habitats may lead to the local extinction of many medicinal plant species. Hence keeping the importance of this tree in view, it was selected for present investigation. 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 plant. Some *In vivo* studies have been carried on *Crescentia alata* but no research has been published on tissue culture aspects. The main objective of this study is to investigate the selection of suitable explants and plant growth regulators on callogenesis and organogenesis in *Crescentia alata*.

Experimental

Materials and Methods

Collection and Authentification of Plant Material

The plant materials were collected from Botanical garden of Department of Botany, JJ Arts and Science college, Pudukkottai, Tamilnadu. It was collected during the monsoon season. Young nodal stem plant (soft, non woody and green in colour) and leaf explants (soft and green in colour) were selected as an explants source. 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 and shoot regeneration. The basal nutrient media MS (Murashige and Skoog) supplemented with different combinations of PGRs (auxin and cytokinin) were used [11].

Explant Surface Sterilization

Freshly harvested young stem and leaves were cleaned and dead/decaying parts separated, that were washed with the running tap water for 30 minutes to remove the dust particles. The explants were surface sterilized with Tween-20 and finally with 0.1% $HgCl_2$ for 5 minute under laminar air flow.

Inoculation of Ex-Plants

Stem and leaf segments about 1-1.5 cm. were prepared aseptically and were implanted vertically on MS medium prepared with different concentrations of auxins and cytokinins, singly or in combination, for callus and shoot induction. The medium containing 3% sucrose was solidified with 8% agar (including different concentration of PGRs). 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 20-25min.

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 16hours. Cultures were visited regularly to observe their response to tissue culture and data were recorded.

Results and Discussion

Callus Induction

Leaf and nodal explants are used for callus induction on MS medium supplemented with different combinations of auxin and cytokinin. Different concentration of different auxins (2,4-D, IAA, NAA and IBA) in combination with cytokinin (BAP). Within 10-20 days of inoculation, callus induction started at the cut end of the explants. The maximum amount of callus induction was observed from BAP 2 mg/l + IBA 1 mg/l+ IAA 0.5mg/l from leaf explants (Table 1, Figure a & b). The colour of the callus was yellowish green which indicates the plant cells undergo stress when inoculated on MS nutrient media in culture condition. The rest of combinations of auxins and cytokinins were capable of producing more or less poor results. Both auxins and cytokinins are major growth regulators that have profound influence on various phenomenons of cell division, callus induction and regeneration. Previous reports have also shown that the regeneration frequency was higher in combination of BAP and NAA in *Aristolochia indica* and *Abelmoschus moschatus* [12,13]. Plant hormones are among the most important physiological factors affecting the callus growth of plants *in vitro*. The effect of plant growth regulators (PGRs) on callus induction and growth of different plant species were studied in several research reports. In this respect, similar response in the callus formation and shoot multiplication of *Oroxylum indicum* was also observed [14]. The major differences in the response of different plants and different explants to

tissue culture conditions lie in the ratio of auxins to cytokinins [15]. The auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and auxins play an important role in the callus induction and different types of auxins had various effects reported by Gang *et al.,* [16]. Both auxins and cytokinins are major growth regulators that have profound influence on various phenomenons of cell division, callus induction and regeneration [17,18].

Table 1. Influence of various PGRs on callus induction in C*rescentia alata* leaf explant

Sr. No.	MS+3%SUCROSE +(mg/l)PGRs	Colour of Callus	Days of Response	Degree of Response
1	1.0 2,4-D	Green compact	25	+
2	2.0 2,4-D	Green compact	25	++
3	3.0 2,4-D	Green compact	25	++
4	4.0 2,4-D	Green compact	25	++
5	5.0 2,4-D	Green compact	25	+
6	1.0 BAP	-	-	-
7	2.0 BAP	-	-	-
8	3.0 BAP	-	-	-
s9	4.0 BAP	-	-	-
10	5.0 BAP	-		-
11	1.0 BAP +1.0 IBA + 0.5 IAA	Yellowish green	22	+
12	**2.0 BAP +1.0 IBA + 0.5 IAA**	**Yellowish green**	**20**	**+++**
13	3.0 BAP +1.0 IBA + 0.5 IAA	Yellowish green	20	++
14	4.0 BAP +1.0 IBA + 0.5 IAA	Yellowish green	20	++
15	5.0 BAP +1.0 IBA + 0.5 IAA	Yellowish green	20	++
16	1.0 IAA	-	-	-
17	2.0 IAA	-	-	-
18	3.0 IAA	-	-	-
19	4.0 IAA	-	-	-
20	5.0 IAA	-	-	-
21	1.0 IBA	-	-	-
22	2.0 IBA	-	-	-
23	3.0 IBA	-	-	-
24	4.0 IBA	-	-	-
25	5.0 IBA	-	-	-
26	1.0 2,4-D+1.0Kn	Whitish green	22	+
27	2.0 2,4-D+1.0Kn	Whitish green	22	+
28	3.0 2,4-D+1.0Kn	Whitish green	22	+
29	4.0 2,4-D+1.0Kn	Whitish green	22	+
30	5.0 2,4-D+1.0Kn	Whitish green	22	+

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

Shoot Regeneration

The proliferation of shoots achieved directly from nodal explants and shoots formed was maximum (3-5) with early response on MS medium supplemented with BAP 2mg/l + IBA 1 mg/l+ IAA 0.5mg/l respectively. Direct shoot proliferation from the nodal explant could be adequate amount of cytokinin along with small amount of auxins supplied in medium. As per the data recorded, the highest numbers of shoots were observedin MS medium consisting of BAP 3mg/l + IBA 1mg/l + IAA 0.5mg/l (Table 2, Figure c & d). However, an increase or decrease in the concentrations and combinations of 2,4-D and Kn showed a negative trend in shoot proliferation. Several studies have been reported regarding the effects of plant growth regulators on shoot growth of different plants.The shoot promoting effect of auxin and cytokinin combinations on organogenic differentiation has been well established in several systems [19]. The maximum of shoots were obtained from nodal explant of *portulace grandiflora* at BAP (4.0 mg/l) [20]. In conclusion, our work shows that conservation of *C. alata* is possible by *in vitro* tech-nology, although few accessions were sensitive to *in vitro* conditions and we described a simple *in vitro* regeneration method for *Crescentia alata* that would be helpful for mass propagation of this species.

Table 2. Effect of cytokinins and auxins supplemented in various combinations on nodal segments of *Crescentia alata*

Sr. No.	Concentration of growth regulators (mg/L)			Number of days required for shoots	Number of Shoots (Mean±SD)
	BAP	IBA	IAA		
1	1.0	1.0	0.5	24	2.6±0.84
2	2.0	1.0	0.5	21	2.7±1.05
3	**3.0**	**1.0**	**0.5**	**20**	**3.8 ±1.10**
4	4.0	1.0	0.5	22	2.4 ±0.96
5	5.0	1.0	0.5	25	1.6 ± 0.84

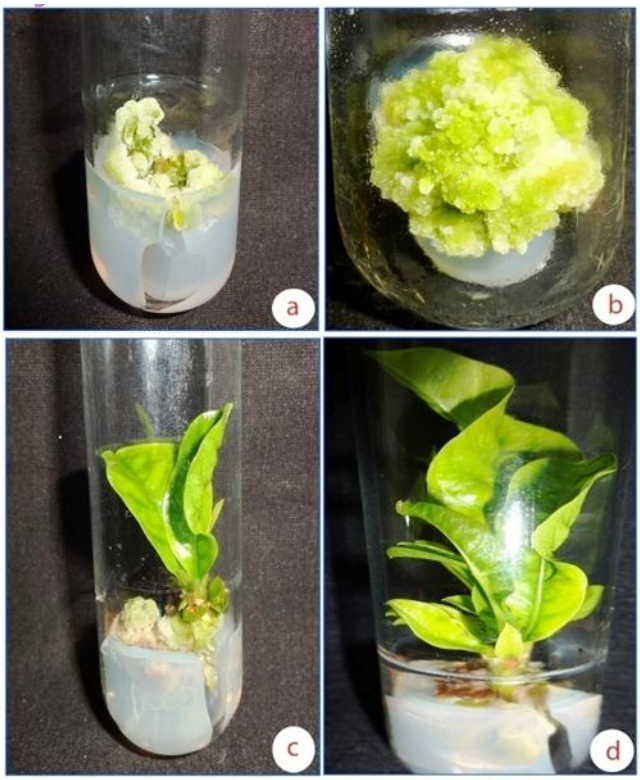

Figure 1. a- Leaf derived callus after 20 days of inoculation, b- Green compact leaf callus after 25 days of inoculation, c- Shoots raised from nodal segments after 30 days of inoculation, d- after 35 days of inoculation.

Conclusion

In vitro culture of plants has gained importance during recent years because this technique can be used for the rapid multiplication and *ex-situ* conservation of some plants. To the best of our knowledge, no report is available about micropropagation of *crescentia alata*. The aim of the present work was to determine the culture conditions for micro-propagation of this plant. The present study discovers nodal segment as effective explants which expressed maximum shoot induction in MS medium with different concentration of PGRs. Further this study showed that the high frequency organogenesis occurs by using BAP, IBA and IAA is possible. It could be used as a tool to protect the biodiversity/natural vegetation of *C. alata*.

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