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Trends in Life Sciences
An International Peer-Reviewed Journal
www.sciencejournal.in
DAMA INTERNATIONAL

IN VITRO CALLUS INDUCTION AND REGENERATION FROM NODAL EXPLANTS OF *ASYSTASIA GANGETICA* (L.) T. ANDERSON

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ABSTRACT
Asystasia gangetica (L) (Acanthaceae) has medicinal and nutritional importance owing to its mineral content and secondary metabolites. This study establishes a method for micropropagation using nodal segments as explants. The effect of auxins and cytokinins were investigated for callus and multiple shoot induction under In vitro conditions using MS medium. The highest frequency of callus induction was noticed with either with 2,4-Dichlorophenoxyacetic acid (2mg/L) alone or in combination of Kinetin (KIN) and α-naphthaleneacetic acid (NAA) (2 +5 mg/L) . The maximum number of shoots (6.4$\pm$ 1.14) was noticed on MS medium + 10 mg /L KIN. The regenerated shoots were transferred to half strength MS medium containing indole-3 butyric acid (IBA; 2.0 mg/L) alone. After hardening, the plantlets were shifted to the green house and subsequently established in the field conditions with 100 % survival rate.The protocol standardized for shoot proliferation and regeneration in *A.gangetica* from nodal segments is suitable for micropropagation and conservation of this medicinal plant.

KEYWORDS: *Asystasia gangetica* , micropropagation , nodal explant , BAP , 2,4-D

INTRODUCTION

Asystasia gangetica (L) (Acanthaceae) commonly known as Chinese violet or creeping foxglove , is a rapidly growing plant . The plant is a spreading herb or ground cover reaching about 60 cms in height or upto 3 m if supported , the stem roots easily at the nodes and this enables it to spread quickly. The leaves are simple and opposite, flowers are purple blue or violet in color. The presence of biologically active terpenoids in the leaves (Ezike *et al.,* 2008) makes this plant an important medicinal plant that is used in several ailments like rheumatism, diabetes, ulcers (Pradeep *et al.,* 2010 ; Tillo *et al.,* 2012) inflammation and cancer (Stewart *et al.,* 2013) . The plant is considered as potential nutritious source with high content of fibers , in addition to minerals like calcium, zinc, magnesium, iron in high percentage (Odhav *et al.,* 2007 ; Yang and Keding, 2009 ; Orech *et al.,* 2013). Protocols for in vitro plant production via direct and indirect morphogenesis have many potential applications and multiple shoot induction using nodal explants have proved successful for rapid propagation of a number of medicinally important species such as *Adhatoda beddomei* (Charantharayil et al., 1994) *Santolina canescens* (Casado *et al.,* 2002); *Cassia angustifolia* (Siddique and Anis, 2007) and *Justicia gendarussa* (Thomas and Yoichiro, 2010). *In vitro* culture allows maintaining clonal fidelity, and also assures the consistent production of true-to-type plants within a short span of time irrespective of season. There are no previous reports of micropropgation of this species, except one report of in vitro propagation of *A.dalzelliana* using leaf disc as explant (Sumana and Kaveriappa, 1996). In this study, we report a rapid and efficient in vitro multiplication and regeneration system of this species using nodal segments as explants, which is the first report of direct regeneration from *A.gangetica*.

MATERIALS AND METHODS:

Explant Preparation and Surface Sterilization:

Nodal segments from the top 2nd- 4th position were collected from actively growing shoots of *Asystasia gangetica*. The explants were thoroughly washed in running tap water for 1 hour, followed by wash in a 1% (v/v) liquid detergent (Labolene, Qualigens) and 1 % Tween -20 for 20 minutes . The plants were then washed thoroughly in running tap water to remove the traces of the detergent. The Explants were then dipped in 70% alcohol for 2 mins and surface sterilized with 0.1% $HgCl_2$ for 5 mins, followed by several washes in sterile water to completely remove the traces of $HgCl_2$. After trimming the cut ends, explants were cut into desirable sizes and inoculated into the culture media under sterile conditions.

Media and Culture conditions:

The nodal explants were cultured on MS basal medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose as carbon source and solidified with 0.8% (w/v) agar as gelling agent, supplemented with various combination of phytohormones ,viz., Auxin (2,4-D , NAA , IBA) Cytokinin (BAP , KIN) in different concentrations ranging from (0.5 - 10 mg/L) either alone or in combination for callus induction and shoot multiplication respectively. Explants inoculated onto MS medium without auxin / cytokinin supplementation were served as controls.The pH of the media

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was adjusted to 5.7 by adding 1M NaOH or 1M HCl prior to autoclaving for 20 mins at 121^0 C for 15 psi. The cultures were maintained in culture room under regular cycle of 16 hrs light and 8 hrs of dark provided by white cool fluorescent tubesat 25 ± 2^0C, with relative humidity of 50-60%.

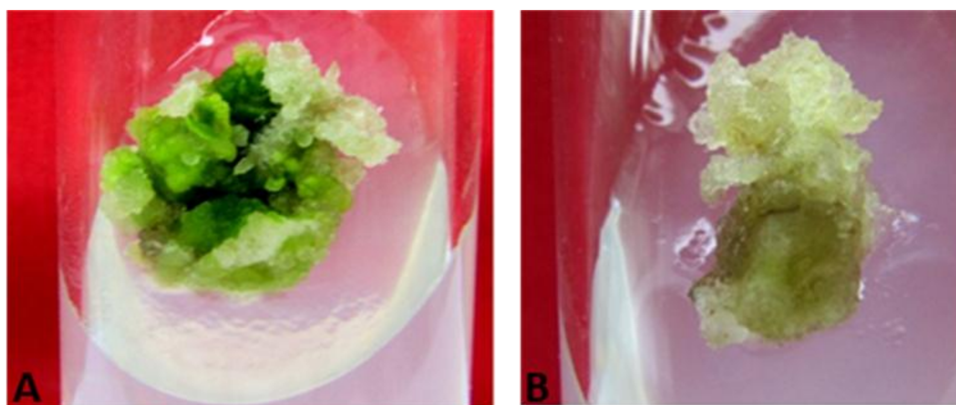

Figure 1 : Different types of callus formed from nodal explants ; A - Six weeks old callus on MS+KIN+NAA (2+2mg/lt) ; B-Two weeks old callus on MS+NAA+2,4-D (5+2mg/lt) .

Table 1. Influence of different concentrations of various Auxins /Cytokinins on callus induction from nodal segment explants of*A.gangetica.*

2,4-D mg/L	KIN mg/L	NAA mg/L	Percentage of explants responded	Callus morphology
1	-	-	50%	CreamishWhite friable callus
2	-	-	100%	
3	-	-	100%	
4	-	-	100%	
5	-	-	100%	
6	-		100%	
2	1	-	100%	Pale green friable rhizogeniccallus
2	2	-	100%	Green hard rhizogenic callus
2	3	-	50%	Green hard rhizogenic callus
2	4	-	0%	No response
2	5	-	0%	-
2	6	-	0%	-
2	7.5	-	0%	-
2	8	-	0%	-
2	-	1	85%	Pale green friable granular callus
2	--	2	85%	Creamish white friable callus
2	-	3	85%	
2	-	4	85%	
2	-	5	100%	
2	-	6	40%	
2	-	7.5	40%	
2	-	8	40%	
2		9	0%	No response
-	1	2	85%	Pale green friable rhizogenic callus
-	1	5	85%	Hard green rhizogenic callus
-	2	5	100%	
	2	5	100%	

Rooting and Hardening of Plantlets:

Regenerated *in vitro* shoots of 2 - 3 cms long were separated from multiple shoot clumps and transferred to half strength MS medium fortified with IBA (1.0 mg/L) to induce roots. Both single and bunches of 3-4 shoots were subjected to root induction. The well rooted plantlets were washed with sterile water and transplanted to small plastic pots filled with soilrite mixture containing vermiculite, peat moss and coco peat (1:1:1 ratio) for hardening and establishment under controlled environmentalconditions for 2 weeks. The transparent plastic bags were inverted over the potted plantlets to maintain high humidity and three weeks after hardening, the plantlets were transferred to field and survival rate was assessed after a month.

Data Recording:

The data on adventitious bud formation and subsequent regeneration were recorded after 3 and 6 weeks of cultures respectively. The percentage of shooting, rooting and root length were recorded after 20 days and 30 days of sub culture respectively on rooting medium. Each value of data represented the mean (±SE) of 5 cultures per treatment.

Figure 2 : Multiple shoot induction from nodal regions , A- Three weeks old culture showing initiation of multiple shoots from nodal explant on MS medium supplemented with IBA (3 mg/lt) , B - Three weeks old culture showing multiple shoots on MS supplemented with BAP (5mg/lt) , C - Six weeks old culture on MS supplemented with KIN (10 mg/lt) , D - Hardened plant after 8 weeks in soilrite. E- Plants acclimatized in soil under controlled conditions.

Table 2. Effect of plant growth regulators on shoot regeneration from nodal segments of *A. gangetica* in MS medium after 8 weeks of culture. Values represent means ± SE.

Growth regulators (mg/L)			% Regeneration	No of shoots/Explant	Shoot length (cm)
BAP	KIN	IBA			
0.1	-	-	50	2.0±0.31	8.40± 0.24
0.3	-	-	50	2.0±0.20	9.00±0.20
0.5	-	-	70	2.0±0.44	7.94±0.34
0.7	-		80	2.2±0.24	8.32±0.70
1	-	-	80	1.8±0.20	9.80±0.48
2	-	-	80	1.2±0.20	10.20±0.37
3	-	-	80	1.2±0.20	9.40±0.50
4	-	-	80	1.4±0.20	10.90±0.33
5	-	-	80	1.6±0.30	9.54±0.17
7	-	-	80	1.4±0.30	10.42±0.16
8	-		80	1.6±0.21	10.60±0.50
-	0.1	-	25	1.8±0.20	8.00±0.47
-	0.3	-	75	1.6±0.24	9.20±0.58
-	0.5	-	75	1.8±0.20	9.70±0.43
-	0.7	-	100	2.4±.33	9.70±0.43
-	1	-	100	2.4±0.40	8.06±0.34
-	2	-	100	3.2±0.24	9.30±0.53
-	4	-	100	2.8±0.20	7.56±0.52
-	5		100	3.6±0.24	9.84±0.35
-	6	-	100	3.6±0.50	9.14±0.21
-	7	-	100	3.2± 0.20	9.20±0.10
-	8	-	100	2.8±0.20	9.20±1.34
-	10		70	6.4±.58	9.22±0.13
-	-	0.1	50	1.2±0.20	8.76±0.18
-	-	0.3	50	1.4±0.24	8.86±0.19
-	-	0.5	50	1.0±0.21	8.70±0.30
-	-	0.7	70	1.0±0.0	8.90±0.78
-	-	1	70	1.2±0.16	7.40±1.03
-	-	2	70	1.0±0.0	10.00±0.70
-	-	3	80	1.4±0.24	8.90±0.33
-	-	4	80	2.6±0.24	9.10±0.29
-	-	5	80	2.8±0.20	8.38±0.50
-	-	7	80	3.6±0.24	9.10±0.11
-		8	80	3.8±0.20	9.28±0.23

RESULTS AND DISCUSSION:

Callus induction: For callus induction from nodal explants, the MS medium supplemented with 2,4-D alone (1-6mg/L) or in combination with KIN (1-10mg/L) NAA (2-10mg/L) was employed .Callus proliferation from the nodal segment occurred within 4 weeks and callus remained creamish white (only 2,4,-D) to pale green friable callus when used in combination. Of the various concentration of 2,4-D tested , 2-6mg/L was found to be

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optimum with 100% response. Callusing was high when 2,4-D (2mg/L) was used in combination of KIN (1-2mg/L). The frequency of callus occurrence on medium decreased when KIN concentration was increased in combination with 2,4-D. No callus formation was observed on medium without growth regulators and with 2,4-D (2mg/L) and KIN (4-10 mg/L) combination. Creamish white friable callus was developed when NAA (1-5mg/L) was used in combination with 2,4-D (Fig 1B) and a maximum response of 40% was observed when concentration of NAA (6-10mg/L) was increased . But, the combination of KIN (2mg/L) and NAA(4-5mg/L) increased the callus induction frequency to 100% (Table 1).The combination of NAA(2-5mg/L) and KIN (1-2mg/ L) induced pale green friable callus . Rhizogenesis was also noticed in few cases (1-2mg/ L) but at higher concentration, rhizogenesis completely diminished giving rise to highly proliferating callus (Fig.1 A). The calli was separated from primary explants and transferred onto MS medium containing KIN and NAA for regular sub culturing. Callus formation can be attributed to exogenous addition of 2, 4-D, KIN and NAA. The addition of KIN to 2,4-D induced hard rhizogenic callus but KIN concentration of more than 4 mg/L was found to be unsuitable for callus induction when used in combination with 2,4-D. Higher kinetin concentration was found to be antagonistic for callus proliferation and no organogenesis was seen even after maintaining the callus for three months.

Regeneration from nodal segments: Direct shoot induction bypassing callus phase is considered to be advantageous as it overcomes somoclonal variation. We tested the direct regeneration from nodal segments by treating them with two cytokinins along with auxin IBA either alone or in combination .The nodal explants did not show any regeneration when cultured on MS medium free from growth regulators even after 30-45 days. However, concentration and type of cytokinin significantly affected the percentage of shoot regeneration and shoot length. Of the two cytokinins (viz BAP and KIN) tested , KIN was more effective than BAP in inducing shoots from nodal segments (Table 2). Similar results have been reported in *Brugmansia suaveolens* (Shekhawat, 2012). The percentage of shoot response increased with increased concentration of KIN up to 10mg/L (Fig 2 C) , which induced 100% explants with shoot length varying from 9-10 cms within 8 weeks (Table 2). It took approximately 21 days for initial shoot formation with two leaves irrespective of treatments, though in some cases there was callusing at the base.). Gnanaraj (2012) has reported highest percentage (93.60±0.71) of shootlets formation for *A. aspera* and (94.70±0.53) percentages for *A. bidentata,* with maximum number of shoots/explants (10.60±0.36) and (9.50±0.56) respectively in MS medium fortified with 5.0 mg/L of BAP. Highest number of shoots (6-7) were noticed at higher concentration of KIN (10mg/L) although the percentage response decreased to 70%. Jeyachandran (2012) observed a maximum shoot responses (80%) when treated with KIN at 1.0 mg/L in *Dipteracanthus prostratus.* Cytokinins are considered to be most essential for shoot induction and elongation in several medicinal plants including *Mucuna pruriens* (Faisal et al.,2006), *Aristolochia indica* (Pattar and Jayaraj, 2012). Further, we tested the effect of auxin IBA either alone or in combination with BAP on shoot regeneration from nodal explants. When IBA was used alone in the range of 0.1 to 10mg/L, within two weeks shoot was induced (Fig 2A). The percent response was slow with up to 70% at lower concentration but it increased to 80-90% along with the increased shoot numbers (4-5) at higher concentration of IBA. Rooting was observed only in the fifth week onwards and subsequently there was a robust elongation of shoots and roots without need for any subculture.

Rooting and Hardening of Plantlets:
An efficient routing protocol is always necessary to obtain whole plants from regeneration. The shoots regenerated from nodal segments using BAP and KIN were directly transferred to root induction medium containing half strength MS medium containing 2mg/L IBA which was more effective, as it induced most of the shoots to form roots within a week (data not shown). After two weeks of culture in this medium, an average of 2-3 roots were induced, each with an average of 5.5 ± 1.5 cm length. Stimulation of rooting by IBA in this study is similar to findings in other medicinal plant species like *Ceropegia candelabrum* (Beena *et al.,* 2003)*, Chlorophytum arundinaceum* (Lattoo *et al.,* 2006) , *Aerva lanata* (Sahu *et al.,* 2013) and *Scabiosa* (Wang *et al.,* 2013).

Shoots grown on IBA didn't require any sub culturing and were directly hardened on soilrite (Fig 2 D) for 15 days. Plantlets with three to four internodal regions along with leaves and well developed roots were successfully acclimatized in earthen pots containing a 3:2:1 (v/v/v) mixture of garden soil, vermiculite, sand (Fig 2 E) and eventually established in soil. The regenerated plants showed robust growth without any morphological variations with 100% survival rate. In summary, we have developed a regeneration system for *A. gangetica*, which enables facile in vitro propagation of plants. To our knowledge the present study is the first report for in vitro shoot regeneration of *A. gangetica* from nodal explants. The procedure described here provides a rapid and prolific regeneration system using individual cytokinins and their successful rooting and acclimatization. It is significant to note that all the multiple shoots have been induced when growth regulators are used alone and not in combination. It is worthwhile to make

detailed study of the effect of cytokinins when used in combination and their effect on bioactive metabolite production which is underway.

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