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In vitro Rapid Clonal Propagation of *Aristolochia bracteolata* Lam. (Aristolochiaceae)-A Valuable Medicinal Plant

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Abstract: Propagation of *Aristolochia bracteolata* relies only on which seed has low viability. For this reason, the natural propagation of this species is hampered. In this consequence an efficient regeneration protocol through *in vitro* direct organogenesis was developed. The shoot bud induction and shoot multiplication was significantly higher in nodal segments as compared to shoot tip and axillary bud. Maximum number of shoots (6.5) with high frequency of shooting response (100%) was obtained in nodal explants cultured on Murashige and Skoog medium (MS) fortified with 4.0 mg/l of 6-benzylaminopurine (BAP). Then the BAP (4.0 mg/l) combined with NAA 0.5 mg/l (Naphthalene acetic acid) was found to be more suitable for getting more number of shootlets (8.9). High frequency organogenesis and multiple shoot regeneration were induced then the microshoots were isolated from the *in vitro* proliferated cluster of shoots and they produced roots on Murashige and Skoog medium supplemented with 0.3 mg/l Indole-3-butyric acid (IBA). The rooted plantlets were transferred for hardening.

Key words: *Aristolochia bracteolate* · Clonal propagation · Microshoots · Shoot tip · Axillary bud and nodal explants

INTRODUCTION

Plants are important source of medicines and play a key role in World health [1]. Almost all cultures from ancient times to today have used plants as medicines. Today medicinal plants are important to the global economy [2]. Approximately 85% of traditional medicine preparations involve the use of plants or there extracts [3]. In the past few decades there has been a resurgence of interest in the study and use of medicinal plants in health care and in recognition of the importance of medicinal plants to the health system [4-6]. This awakening has led to a sudden rise in demand for herbal medicines, followed by a belated growth in International awareness about the dwindling supply of the world medicinal plants [7]. Most of the pharmaceutical industry is highly dependent on wild populations for the supply of raw materials from which medicinally important compounds are extracted.

The genetic diversity of medicinal plants in the world is getting endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines, with little or no regard to the future. Also, extensive destruction of the plant rich habitat as a result of forest degradation, agricultural encroachment, urbanization, etc., are other factors. Hence there is a strong need for proactive understanding in the conservation, cultivation and sustainable usage of important medicinal plant species for future use.

Aristolochia bracteolata is an important medicinal plant of the family Aristolochiaceae. This plant is common in India. Aristolochia is a genus of evergreen and deciduous woody vines and herbaceous perennials. The leaves, roots and fruits of which find diverse uses in indigenous systems of medicine. Roots are anti-inflammatory, diuretic and cardiotonic. It contains an important alkaloid aristolochin ($C_{17}H_{19}O_3N$). Leaves are used to treat cholera and intermittent fever in children. Seeds are a good remedy for inflammation, biliousness and dry cough [8]. Indiscriminate over exploitation of the plant for diverse medicinal uses has resulted in its rarity in nature, which calls for newer approaches for the rapid propagation of this important medicinal plant. The micropropagation of this species has not been reported so far. This work, therefore emphasize the *in vitro* responses achieved in *Aristolochia bracteolata* in terms of direct regeneration from meristematic explants.

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MATERIALS AND METHODS

Different explants were collected from the mature plants grown in the Garden of Jamal Mohamed College, Tiruchirappalli. They were then washed under continuous flashing of running tap water for 30 min treated with a solution of the Teepol (5% v/v) for 10 min and surface sterilized with $HgCl_2$ (0.1% w/v) for 5 min. Finally, the material was washed 5 times with sterilized distilled water to remove any trace of $HgCl_2$. The shoot tip, axillary bud and nodal segments were excised from the disinfected plantlets. The explants were approximately 1.0-1.5 cm in size. The Murashige and Skoog (MS) medium [9] was used for all the experiments and its containing 3% sucrose, 0.8 % agar and supplemented with BAP and 6-furfuryl aminopurine (Kn), either individually or in different combinations with auxins (NAA and IAA). The media were adjusted to pH 5.6 ± 0.2, autoclaved at 121°C for 15 min duration. Cultures were incubated at 25°C with a photoperiod of 16 h at 2000 lux of cool white fluorescent light. Cultures were initiated in 25mm glass tube and sub-cultured regularly on fresh medium at four week intervals in the glass tube. The multiple shoots were isolated and it was transferred into the rooting medium. For root induction the MS medium supplemented with IBA, NAA and IAA in different concentration. Root number and length were recorded after 30 days in culture. The values calculated by Mean ± SD based on 20 replicates per treatment. Developed plantlets were removed from the agar surface, washed and planted in small cups with a mixture of soil, garden soil and farmyard manure (1:1:1 ratio). The plants were covered with polytene bags to maintain the optimal humidity for 15 days. After 2-3 weeks, the plants were taken onto the field.

RESULTS

Regeneration of Plantlets from Shoot Tip Explants: Multiple shoots derived from shoot tip explant cultured on MS medium supplemented with various concentration of BAP were helpful in inducing the growth of meristem tips. However, the best and rapid growth was observed on MS medium supplemented with BAP 4.0 mg/l. At this concentration, the number of shoots as well as length of shoots per explant was found to be 4.4 \ and 6.2 cm respectively. However, maximum numbers of multiple shoots were obtained in BAP combined with a low concentration of NAA. The best response was achieved at 4.0 mg/l BAP + 0.5 mg/l NAA. At this concentration, all the explatns shows a high proliferation multiple shoots after 10 days. The highest mean number of shoots and shoot length per culture were 5.3 and 6.7 cm respectively (Table 1).

Regeneration of Plantlets from Nodal Explants: The nodal explants underwent direct organogenesis when cultured in MS medium using various concentrations of BAP (2.0-10.0 mg/l) alone or combined with various low concentrations of Auxins, IAA and NAA (0.1-2.0 mg/l). It was observed that the shoot induction was more with BAP. The effective concentration for better shoot induction was found to be 4.0 mg/l of BAP. The BAP combined with 0.5 mg/l NAA number of shoots as well as length of shoots per explant was 8.9 and 7.4 cm, respectively (Table 1). BAP is considered one of the most useful cytokinins for the multiplication of nodal explants. It was also found that the number of shoots per culture was increased with the periodic subculture.

Regeneration of Plantlets from Axillary Bud Explants: Direct organogenesis from axillary bud explants was cultured on MS medium supplemented with various concentrations of BAP (2.0-10.0 mg/l) alone or in combination with various low concentrations of IAA and NAA (0.1-2.0 mg/l). It is observed that BAP was more effective for shoot induction. Among the different treatment of BAP, the concentration 4.0 mg/l of BAP gave better response. In this concentration, 100% explants induced to develop shoots. The number of shoots as well as length of shoots per explant was recorded 6.0 and 6.4 cm, respectively (Table 1), reached to 7.3 and 6.8 with 4 mg/l BAP + 0.5 mg/l NAA when Kn supplemeted alone to the media, there was no positive response to multiple shoot but full callus was formed with all concentrations.

Rooting and Acclimatization: The *in vitro* multiple shoots were sub-cultured to develop whole plants for root induction in media supplemented with different concentration of NAA, IAA and IBA. When the rooting media were supplemented with IBA concentration 0.3 mg/l the number and length of roots greatly increased and then decreased at 0.5 mg/l (Table 2). The medium supplemented with NAA or IAA (0.1-0.5 mg/l) had poor rooting. Though using NAA and IAA influence the callus at the basal shoots.

Acclimatization, *in vitro* plantlets were transferred to pots containing mixture of soil, garden soil and farmyard manure (1:1:1 ratio) and healthy roots appeared after two weeks. The survival rate of the clones was about 95%. The plants were hardened for 10-15 days before being transferred to the greenhouse.

The new design of Table 1:

Plant growth regulators (mg/l)				% of shoot	No. of multiple shoot Mean± SD			Average shhot length Mean± SD			Callus
BAP	Kn	IAA	NAA	response	Shoot tip	bud	node	Shoot tip	bud	node	initiation cm
2.0											
4.0											
6.0											
8.0											
10.0											
	2.0										
	4.0										
	6.0										
	8.0										
	10.0										
4.0		0.1									
		0.5									
		1.0									
		1.5									
		2.0									
4.0			0.1								
			0.5								
			1.0								
			1.5								
			2.0								

Table 1: Effect of BAP combined with various concentration of IAA, NAA for multiplication of three different explants of *Aristolachia bracteolate*

Hormone	Conc. mg/l	Total no. of explant	No. of explant responded	% of response	No. of multiple shoot in shoot tip explant (Mean ± SD)	No. of multiple shoot in axillary bud explant (Mean ± SD)	No. of multiple shoot in node explant (Mean ± SD)
BAP	BAP						
	2.0	10	9	90%	3.8 ± 0.9	4.7 ± 1.0	5.7 ± 1.1
	4.0	10	10	100%	4.4 ± 1.1	6.0 ± 1.1	6.5 ± 0.8
	6.0	10	8	80%	3.3 ± 0.6	5 ± 1.0	6.1 ± 1.2
	8.0	10	8	80%	3 ± 0.7	4.1 ± 1.2	5.1 ± 1.2
	10.0	10	7	70%	2.2 ± 1.0	3.8 ± 0.7	4.8 ± 0.6
Kn	Kn						
	2.0	10	-	-	-	-	-
	4.0	10	-	-	-	-	-
	6.0	10	-	-	-	-	-
	8.0	10	-	-	-	-	-
	10.0	10	-	-	-	-	-
BAP+IAA	BAP+IAA						
	4.0 0.1	10	9	90%	5.1 ± 0.7	5.4 ± 1.0	5.6 ± 0.9
	4.0 0.5	10	10	100%	5.2 ± 1.0	6.0 ± 1.2	6.4 ± 0.9
	4.0 1.0	10	8	80%	4.3 ± 1.3	4.6 ± 0.9	5.3 ± 0.6
	4.0 1.5	10	7	70%	3.4 ± 0.9	4.2 ± 0.9	4.2 ± 1.0
	4.0 2.0	10	7	70%	2.5 ± 0.9	3.2 ± 1.0	4 ± 0.8
BAP+NAA	BAP+NAA						
	4.0 0.1	10	8	80%	4.3 ± 1.3	5.1 ± 1.3	5.5 ± 0.8
	4.0 0.5	10	10	100%	5.3 ± 0.9	7.3 ± 0.6	8.9 ± 0.8
	4.0 1.0	10	8	80%	4.6 ± 1.2	4.6 ± 0.9	5 ± 0.9
	4.0 1.5	10	7	70%	3.4 ± 1.3	4.1 ± 1.0	4.4 ± 0.9
	4.0 2.0	10	6	60%	3.1 ± 0.6	3.4 ± 0.9	3.1 ± 0.8

Table 1: Continue

Hormone	Conc. mg/l	Average length of shoot (cm) shoot tip explant Mean±SD	Average length of shoot(cm) axillary bud explant Mean±SD	Average length of shoot(cm) node explant Mean±SD	Remark
BAP	BAP				
	2.0	5.3 ± 0.9	5.8 ± 0.8	6.3 ± 0.6	-
	4.0	6.2 ± 1.2	6.4 ± 1.1	7 ± 0.7	-
	6.0	6 ± 1.0	6.0 ± 0.7	6.8 ± 0.9	-
	8.0	5.8 ± 1.2	5.8 ± 0.8	5.8 ± 0.8	++
	10.0	5.2 ± 1.1	5.2 ± 0.9	5.1 ± 0.4	++
Kn	Kn				
	2.0	-	-	-	+++
	4.0	-	-	-	+++
	6.0	-	-	-	+++
	8.0	-	-	-	+++
	10.0	-	-	-	+++
BAP+IAA	BAP+IAA				
	4.0 0.1	5.5 ± 1.2	6.2 ± 1.1	6.4 ± 1.2	-
	4.0 0.5	6.6 ± 0.8	6.7 ± 0.2	7.2 ± 0.9	-
	4.0 1.0	6.1 ± 1.0	6.0 ± 0.9	6.9 ± 0.1	-
	4.0 1.5	5.8 ± 0.9	5.9 ± 1.2	5.9 ± 0.4	+
	4.0 2.0	5.6 ± 1.2	5.5 ± 0.6	5.5 ± 0.8	+++
BAP+NAA	BAP+NAA				
	4.0 0.1	5.6 ± 0.7	6.5 ± 0.6	6.6 ± 0.9	-
	4.0 0.5	6.7 ± 0.9	6.8 ± 0.2	7.4 ± 1.2	-
	4.0 1.0	6.2 ± 1.2	6.2 ± 1.1	7.0 ± 1.1	-
	4.0 1.5	5.9 ± 0.2	6.0 ± 0.9	6.8 ± 0.3	++
	4.0 2.0	5.7 ± 1.2	5.9 ± 1.2	5.9 ± 0.6	+++

Note:-No callus, + Basal callus, ++ Moderate callus, +++ Full callus.

Table 2: Effect of auxin on *in vitro* rooting of shoots cultured on MS medium

Hormone	Concentration	Percentage	Mean no of roots	Mean No. of root length	Callus initiation
Control	-	-	-	-	-
NAA	0.1	-	-	-	+
	0.2	-	-	-	++
	0.3	-	-	-	+++
	0.4	-	-	-	+++
	0.5	-	-	-	+++
IAA	0.1	-	-	-	+
	0.2	-	-	-	++
	0.3	-	-	-	+++
	0.4	-	-	-	+++
	0.5	-	-	-	+++
IBA	0.1	50	4.9 ± 0.7	6.0 ± 0.9	-
	0.2	80	6.1 ± 0.9	8.5 ± 1.0	-
	0.3	90	11.1 ± 0.6	10.2 ± 0.7	-
	0.4	70	5.5 ± 1.0	6.5 ± 0.6	-
	0.5	40	3.4 ± 0.6	3.7 ± 0.5	+

Note:-No callus, + Basal callus, ++ Moderate callus, +++ Full callus.

DISCUSSION

Tissue culture techniques are being increasingly used for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened with extinction. So far our knowledge goes no report has been published on *in vitro* propagation of *Aristolochia bracteolata*. Thus an attempt was taken on *in vitro* propagation of this medicinal plant species. In the present investigation, BAP is considered one of the most useful cytokinin for multiplication meristematic tissue explants [10-12] has been reported that the BAP used for propagation of different plants like *Saussurea obvallata, Holostema ada-kodien, Gentiana kurroo.*

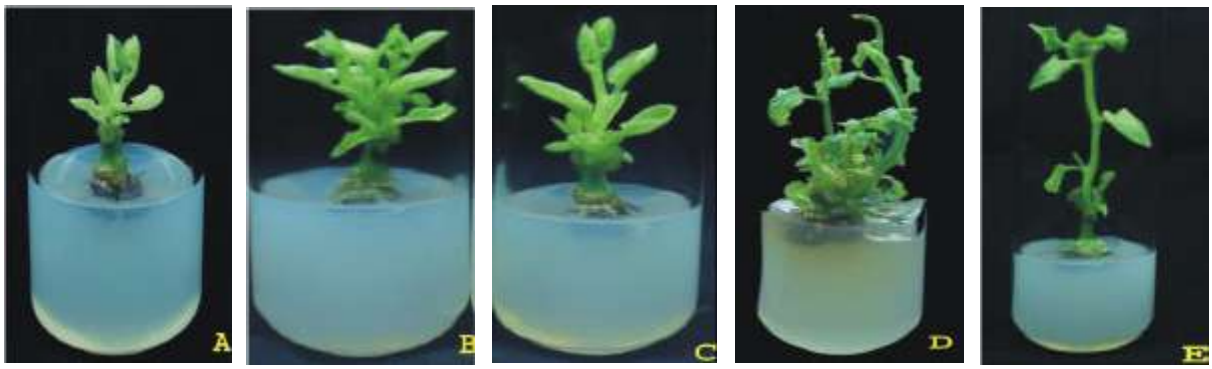

Fig. 1: Effect of BAP combined with various concentration of NAA for multiplication of *Aristolochia bracteolate* using axillary bud explant
A. Multiple shoot initiated from 7th day onwards on MS medium supplemented with 4.0 mg/l BAP+0.5mg/l NAA.
B-D. More than 7 multiple shoots aroused from the second subculture of the explants
E. From the isolated shoots, roots are emerged at the concentration of 0.3mg/l IBA

In the present investigation, combination of BAP with NAA was found more suitable than BAP alone. Among the BAP + NAA supplemented media, the best response was achieved in 4.0 mg/l BAP + 0.5 mg/l NAA (Fig. 1). After 10 days of culture and 100% explants showed high proliferation of multiple shoots in this combinations. The highest mean number of shoots and shoot length (cm) per culture were 8.9 and 7.4 cm, respectively. On the other hand Kn combined with NAA and IAA supplemented media did not give any positive response. Such a combined effects has also been reported in *Petasites hybrids* of family Asteraceae [13]. For example some reference were denoted that the BAP and Kn is combination with different concentrations of NAA and IAA gave better multiple shoots from the meristematic explants [14-18]. For better root induction the hormone IBA (0.3 mg/l) for *Aristalochia bractiolata*. IBA was more resistant than NAA and IAA to degradation in the tissue culture media, both during autoclaving and at room temperature [19]. As with *Wedelia chinensis* [20], this concentration of IBA seems to play a stimulatory role in the process of root formation in *A.bracteolata* shoots. In the IBA medium, the majority of roots developed three weeks earlier than in the NAA and IAA. IBA concentration was beneficial also for both root system development and for shoot quality.

CONCLUSION

In conclusion, an efficient and easy protocol for propagation of the important medicinal plant (*Aristolochia bracteolata* Lam.). This protocol provides a successful and rapid plantlets multiplication technique that can be used for e*x situ* conservation. As a part of domestication strategy, these plants can be grown and further cultivated in destructed and fragmented vegetation. The application of this protocol can help minimize the pressure on wild populations and contribute to the conservation of the valuable flora of the Tamil Nadu, India.

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