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Research Article

In vitro regeneration of plantlets from nodal explants of *Aristolochia saccata* and *Aristolochia cathcartii*

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ABSTRACT

In vitro propagation of *Aristolochia saccata* and *A. cathcartii* were carried out using nodal explant. In both the plants, nodal explants showed direct somatic embryogenesis when cultured on MS medium using various concentrations of BAP (1.0-4.0) and 2iP (1.0-4.0) separately or in combination with low concentration (0.5 and 1.0 mg l^{-1}) of auxin (NAA). It was observed that BAP in combination with NAA was more effective for shoot induction than the hormones used separately. Among different combinations of NAA and BAP, 3.0 mg/l BAP + 1.0 mg/l NAA showed better response in case of *A. saccata* of about 96%, whereas, in *A. cathcartii*, the best response was achieved in 4.0 mg/l BAP + 0.5 mg/l NAA after 28 day of culture and 88.3% explants showed proliferation in this combination. The auxins NAA and IBA were used singly to induce rooting from in vitro raised shoot lets. A range of concentration was tested (0.1, 0.5, 0.8 and 1.0 mg/l) for rooting. In the present study 1/2 strength MS basal medium and the two different auxins (NAA and IBA) were tried, the maximum results on rooting were obtained on half strength with IBA (0.5 mg/l) then NAA.

Keywords: *Aristolochia saccata*; *A. cathcartii*; *In vitro* regeneration; Nodal culture; MS medium.

1. INTRODUCTION

From the time immemorial, plants have been widely used as curative agents in traditional medicines for variety of ailments. In India about 2,500 plants species belongs to more than 1000 genera are being used in the indigenous system of medicine [1]. Northeast India including Assam is represented by about 130 different tribes out of total 427 of India having their own traditional practices. Many herbal remedies individually or in combination have been recommended for the cure of different diseases in traditional medicinal practices by the ethnic communities of Northeast India.

*Aristolochia (*Aristolochiaceae) is an important genus widely used in traditional medicine [2]. During the past two decades, this genus has attracted much interest and has been the subject of numerous chemical and pharmacological studies. It is a rich source of aristolochic acids, which are unique to this genus, and of terpenoids [3].

The family Aristolochiaceae comprises of 8 genera and 450-600 species. The members of the genus *Aristolochia* are mostly distributed in tropical, subtropical, and Mediterranean regions of the world [4-7]. The species of *Aristolochia* are shrubby or perennial herbs, usually climbing.

The genus *Aristolochia* consists of a large number of species, cultivated as ornamentals [8] and popularly used as sources of abortifacient, emmena-

gogue, sedative, analgesic, anticancer, anti-inflammatory, anti-feedant, muscle relaxant, antihistaminic, and antiallergic drugs, for intestinal worms, in the treatment of cholera, stomach ache, abdominal pain, rheumatism, malaria, wounds and skin diseases, and also useful in treatment of different types of poisonous bites and stings [2-3].

The genetic diversity of medicinal plants in the world is becoming endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines. Further, extensive destruction of the plant rich habitat as a result of forest degradation, agricultural encroachment, urbanization, etc., are also some important factors [4]. Hence, there is a strong need for proactive understanding in the conservation, cultivation and sustainable usage of important medicinal plant species for future use. Hence the present study was aimed to develop an effective, reproducible, and simple and improved protocol for in vitro propagation by using nodal explants to make two species of *Aristolochia* viz. *A. saccata* Wall. and *A. cathcartii* Hook.f. & Thomson, throughout the year for pharmaceutical use and also for conservation.

2. MATERIALS AND METHODS

2.1. Plant material and explants sterilization

Two species of *Aristolochia viz., Aristolochia saccata* and *Aristolochia cathcartii* were collected from Karbi Anlong district (25°54′20.22″ N, 93°39′41.16″ E) of Assam, India. The collected experimental plants were grown and maintained in the experimental garden of Botany Department, Gauhati University. Nodal segments from the source plants were used as explants. The explants were coarsely trimmed to a size of 3 cm and washed thoroughly under running tap water for 10 min and then treated with liquid detergent [5% (v/v) Tween-20] for 15 min. Later these explants were washed with double-distilled water for 10 min. The explants were then sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min and washed several times with sterile H_2O to remove all traces of $HgCl_2$. After a final wash, the explants were spread on the pre-sterilized petridishes lined with sterile blotting paper inside a laminar airflow chamber. They were then trimmed finely to the appropriate size (1-1.5 cm). The sterilized explants were inoculated onto solid basal MS medium [9], with different concentrations and combinations of 2iP, BAP and NAA for in vitro regeneration of plants.

2.2. Culture media and growth condition

The stock solution in the required quantity was pipetted out into a standard flask containing distilled water. Sucrose 3% along with 100 mg/l myoinositol were added and dissolved in the media. All the plant growth regulators; additives for the different combinations were added before making up the media to the required volume. pH was adjusted at 5.8 using 0.1 N NaOH or 0.1 N HCl. For solidification, 0.8% w/v agar (HI-MEDIA Lab. India) was then added to the medium and mixed well**.** The sterilization of the culture medium was carried out in an autoclave for 20 min at 121°C and 15 psi pressure. The medium was then poured into pre-sterilized culture vessels, 15 ml was taken in culture tubes (150 × 25 mm), 50 ml was taken in culture bottles and 100 ml was taken in petri plates (150 x 20 mm) under aseptic conditions in a laminar air-flow cabinet.

Nodal segments (1-1.5 cm) were dissected out and all the inoculation operations were carried out under strict aseptic condition inside a Laminar Air Flow chamber, which was made sterile by the incessant exposure of germicidal UV rays for half an hour before use. All operations were carried out using pre-sterilized instruments and glassware. Explants were then aseptically introduced into culture vessels. The culture tubes were then plugged tightly with non-absorbent cotton plugs and the culture bottles and petriplates were sealed tight with sealing film. All cultures were incubated under irradiance of 70 µmol m^{-2} s^{-1} for 16 hour photo-period and temperature of 25±1°C and with a relative humidity of 55-60%.

2.3. Induction of callus and regeneration of plantlets

Basal medium supplemented with different concentrations of 2-isopentenyl-adenine (2-iP) (1.0, 2.0, 3.0, 4.0 mg/l) and benzylaminopurine (BAP) (1.0, 2.0, 3.0, 4.0 mg/l) individually and in combi-

nations with naphthaleneacetic acid (NAA) (0.5, 1.0 mg/l) were tested for the induction of callus and regeneration of shoot and root from nodal explants.

Sub-culturing was done at 14 day intervals onto fresh medium for 6 weeks to induce in vitro regeneration of shoot. Shoot buds were further cultured for elongation in the same medium supplemented with low concentration of cytokinins. The responses of each explant with regard to the induction of shoots, the length of shoot and the percentage of response were recorded after 6 weeks in culture.

2.4. In vitro rooting

In vitro regenerated shoots were rooted on half strength medium supplemented with different concentrations of auxins (NAA and IBA) alone. The response of each explant with regard to the number of roots induced and root lengths per shoot after 2 weeks in culture were recorded.

2.5. Hardening and acclimatization

In vitro grown plantlets were gently removed from culture tubes and washed with slightly warm ($37^{\circ}C$) sterile double distilled H_2O to remove all traces of nutrient medium. After removing media, plants were dipped in 1% w/v solution of bavistine to prevent any fungal infection to newly developed plants. After bavistine treatment the plantlets were carefully planted in plastic pots containing soilrite. The plantlets were irrigated by sprinkling with 0.5 x MS inorganic salts for three to four times per day for seven days. Plantlets were acclimatized for two weeks in an aseptic culture room under (16 h photoperiod at 28 ± $2^{\circ}C$; 8 h in dark at 25 ± $2^{\circ}C$) conditions. Further, the plantlets were exposed gradually to sunlight for acclimatization and were maintained in a garden.

2.6. Data collection and statistical analysis

Data for the percentage of response per explants with different concentrations and combinations of cytokinins and auxins with basal MS medium (shoot regeneration, shoot lengths, number of roots and root lengths) were recorded. Thus obtained data were analyzed statistically using SPSS 16.0 software (IBM Corporation SPSS, North America).

3. RESULTS

3.1. Direct somatic embryogenesis from nodal explants

Regeneration potential of nodal segments was explored on MS medium supplemented with various plant growth regulators and results are summarized in Tables 1 and 2. Nodal segment explants remained green and fresh but failed to develop multiple shoots in growth regulators free MS medium (control). All nodal explants cultured on MS medium supplemented with various concentrations of 2iP and BAP individually and in combination with NAA have developed healthy shoots. Nodal explants cultured on MS medium fortified with cytokinins alone induced multiple shoots at a lesser frequency compared to the media supplemented with combination of cytokinin and auxin (Fig. 1 and Fig. 2).

All the concentrations of BAP and 2iP facilitated shoot bud differentiation but BAP being more efficient than 2iP in terms of percent regeneration, number of shoots and shoot length. Among the various concentrations of BAP and 2iP tested, 3.0 mg/l BAP showed the highest shoot regeneration frequency of 86.6 ± 2.8%, the highest number of shoot were recorded as 1.8 ± 0.34 in *A. saccata*, but the highest shoot length (4.34 ± 0.07 cm) was observed at reduced concentration of BAP (1.0 mg/l).

In case of *A. cathcartii*, 4.0 mg/l BAP showed the highest shoot regeneration frequency of 73.3 ± 2.8% and shoot number (3.8 ± 0.45), but the highest shoot length (4.02 ± 0.1 cm) was observed at 2.0 mg/l concentration of BAP.

The synergistic influences of auxins with cytokinins was evident when combination of optimal concentration of each cytokinins with different concentrations of NAA (0.5 and 1.0 mg/l) were tested (Tables 1 and 2). Addition of NAA markedly enhanced the percent regeneration and number of shoots for both the *Aristolochia* sp. used for *in vitro* propagation. Among all the cytokinin and auxin combinations, the maximum percent regeneration in

A. saccata was found as 96.6 ± 2.8% and number of shoots (3.4 ± 0.55) per explants were obtained at 3.0 mg/l BAP + 1.0 mg/l NAA. But the highest shoot length (4.02 ± 0.08 cm) was recorded at the combination of 1.0 mg/l BAP + 0.5 mg/l NAA.

In case of *A. cathcartii*, the maximum percent of regeneration was recorded as 88.3 ± 2.8% and number of shoots (6.2 ± 0.44) at 4.0 mg/l BAP + 0.5 mg/l NAA. Here, the highest shoot length (3.82 ± 0.1 cm) was recorded at the combination of 2.0 mg/l BAP + 1 mg/l NAA.

3.2. *In-vitro* rooting

The *in vitro* raised shootlets were sub cultured on ½ strength MS medium augmented with 0.1-1.0 mg/l either NAA or IBA for both *A. saccata* and *A. cathcartii* for root formation. At 14th day, the *in vitro* raised shootlets were produced *in vitro* rootlets without any callus proliferation. Medium containing 0.5 mg/l of IBA was proved to be the most effective for rooting of micro shoots than that containing any other concentrations of NAA in case of both the plants evaluated (Tables 3 and 4). Here, NAA did not significantly improve the parameters evaluated. Highest percentage (83.3 ± 2.8%), maximum number of rootlets/shootlet (2.8 ± 0.44) and mean length of rootlets (3.22 ± 0.16 cm) were observed in in *A. saccata.* In *A. cathcartii*, the medium containing 0.5 mg/l of IBA, highest rooting was observed (78.3 ± 2.8%) percent shoots induced rooting within 14 days of culture and the mean number of root per culture and root length was recorded as 3.8 ± 0.44 and 3.04 ± 0.08 cm respectively.

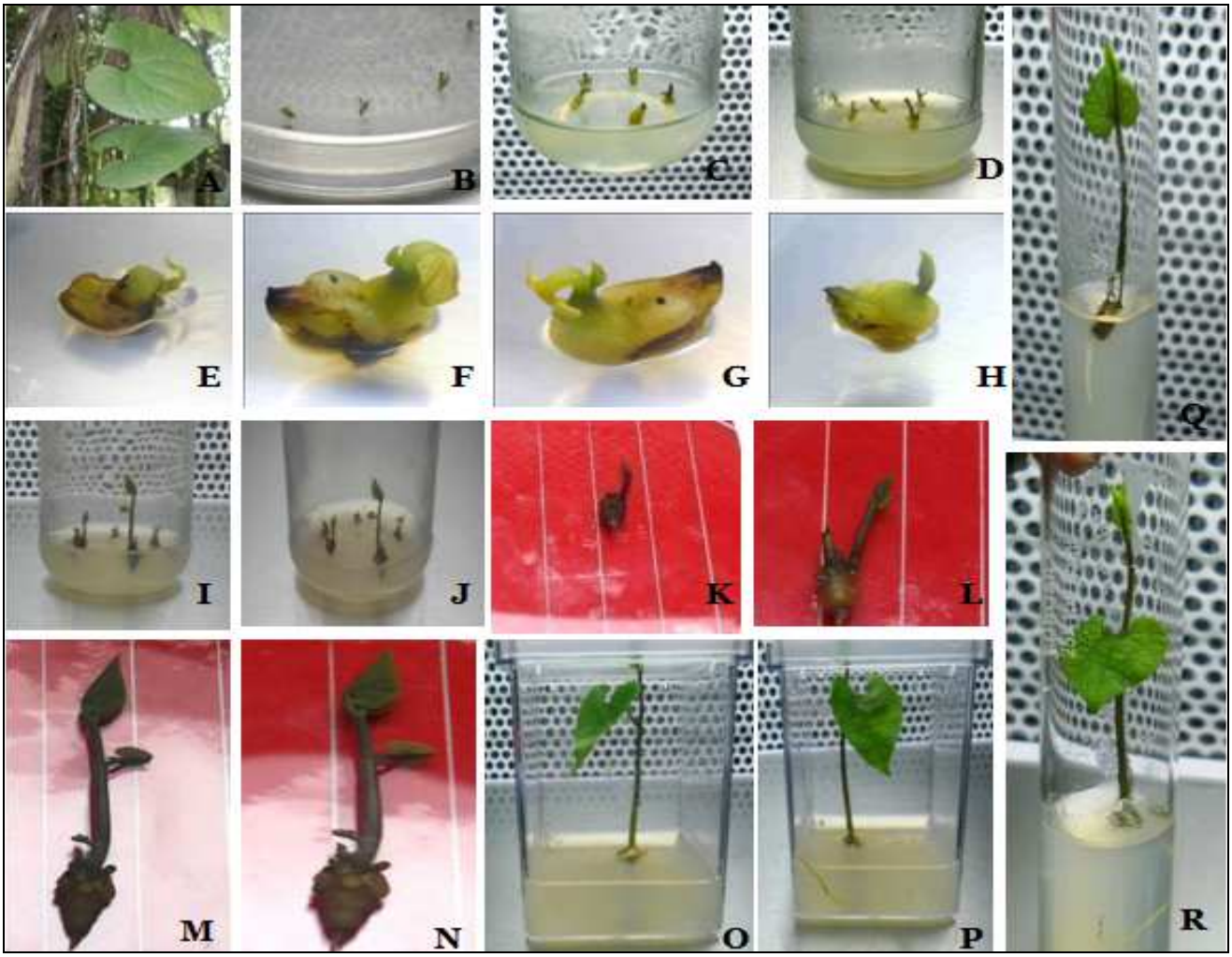

Figure 1. Different stages of *in-vitro* regeneration of *A. saccata* from nodal explant. **A** = plant in wild condition, **B** = inoculation of nodal explants in MS medium, **C-D** = initial days after inoculation in MS medium, **E-H** = direct organogenesis from explant in 3:1 mg/l of BAP and NAA in MS medium, **I-L** = multiple shoot regeneration in 3:1 mg/l of BAP and NAA, **M-N** = shoot elongation in 1 mg/l BAP, **O-R** = stages of rooting in 0.5 mg/l IBA in ½ MS medium.

Table 1. Effect of cytokinins and auxins individually and in combinations for organogenesis from nodal explants of *A. saccata* (after 6 weeks).

Plant growth regulators (mg l^{-1})			Response of nodal explants (%)	Number of shoots/ explant (Mean ± SD)	Shoot length/explant (cm) (Mean ± SD)
2iP	BAP	NAA			
Control (PGR free)			-	-	-
1			0	0	0
2			71.6±2.8	1.2±0.44	3.70±0.08
3			76.6±2.8	1.6±0.45	3.21±0.04
4			61.6±2.8	1.2±0.45	2.48±0.05
1		0.5	0	0	0
2		1	75.0	1.6±0.34	3.26±0.10
3		1	88.3±2.8	2.2±0.34	2.80±0.04
4		0.5	66.6±2.8	1.4±0.24	2.34±0.07
	1		80.0	1.6+±0.54	4.34±0.07
	2		73.3±2.8	1.4 ± 0.53	3.88±0.05
	3		86.6±2.8	1.8±0.34	3.38±0.10
	4		70.0	1.2±0.44	2.90±0.04
	1	0.5	88.3±2.8	2.2±0.44	4.02±0.08
	2	1	81.6±2.8	2.6±0.54	3.68±0.10
	3	1	96.6±2.8	3.4±0.55	3.38±0.11
	4	0.5	71.6±2.8	2.8±0.44	2.82±0.04

Data mean of 3 replicates ± S.D.

Table 2. Effect of cytokinins and auxins individually and in combinations for organogenesis from nodal explants of *A. cathcartii* (after 6 weeks).

Plant growth regulators (mg l^{-1})			Response of nodal explants (%)	Number of shoots/ explant (Mean ± SD)	Shoot length/explant (cm) (Mean ± SD)
2iP	BAP	NAA			
1			0	0	o
2			31.6± 2.8	3±0.82	3.6 ± 0.2
3			33.3± 2.8	3.25±0.50	3.24 ± 0.1
4			36.6± 2.8	3.5±0.57	2.98 ± 0.14
1		0.5	0	0	0
2		1	33.3± 2.8	3.2±0.45	3.11 ± 0.2
3		1	41.6± 2.8	3.6±0.50	3.06 ± 0.2
4		0.5	43.3± 2.8	3.8±0.42	3.02 ± 0.21
	1		0	0	0
	2		41.6± 2.8	3.4±0.54	4.02 ± 0.1
	3		43.3± 2.8	3.6±0.57	3.72 ± 0.08
	4		73.3± 2.8	3.8±0.45	3.42 ± 0.25
	1	0.5	0	0	0
	2	1	73.3± 2.8	4±0.70	3.82 ± 0.1
	3	1	81.6± 2.8	4.2 ±0.44	3.54 ± 0.25
	4	0.5	88.3± 2.8	6.2±0.44	3.26 ± 0.2

Data mean of 3 replicates ± S.D.

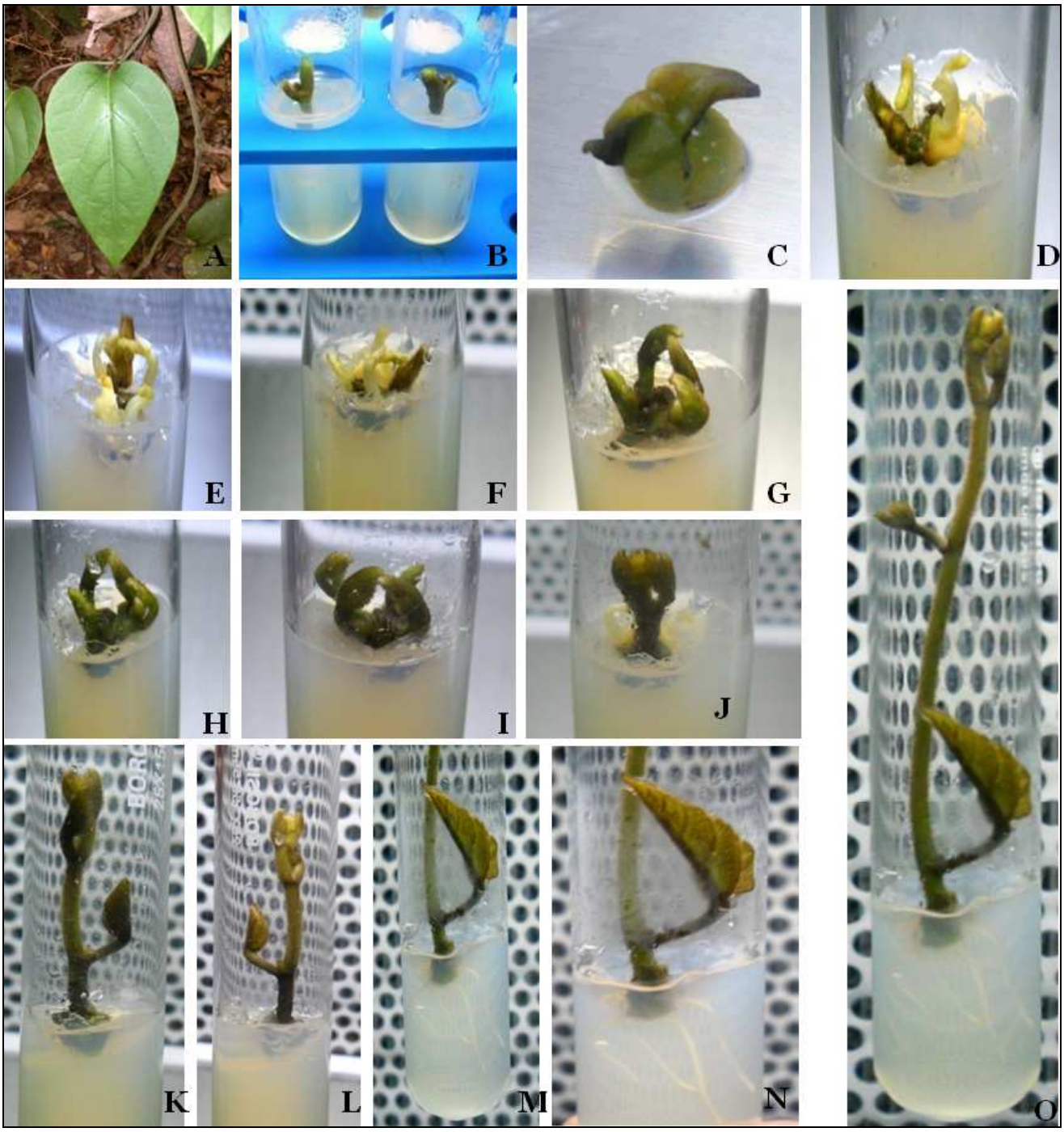

Figure 2. Different stages of *in-vitro* regeneration of *A. cathcartii* from nodal explant. **A** = plant in wild condition, **B** = inoculation of nodal explants in MS medium containing 4.0 mg/l BAP and 0.5 mg/l NAA, **C** = shoot bud initiation from an explant, **D-I** = direct organogenesis and multiple shoot regeneration in 4:0.5 mg/l of BAP and NAA, **J** = individual shoot bud transferred to shoot elongation medium containing 2 mg/l BAP, **K-L** = shoot elongation in 2 mg/l BAP, **M-O** = stages of rooting in 0.5 mg/l IBA in ½ MS medium.

3.3. Acclimatization and hardening

The rooted plantlet were successfully hardened off inside the growth room in sterile soilrite for 2 weeks and eventually established in natural soil. There was no detectable variation among the potted plants with respect to morphological and growth characteristics (Fig. 3).

After 15 days, *in vitro* raised plantlets were hardened in polycups with soilrite, irrigated with 0.5× MS liquid medium. The plants were kept in a culture room for 14 days. Approximately,

75% of plants were successfully established in polycups for both the experimental plants. After 14 days the polycups hardened plants were transferred to pots placed in and kept in poly house. Sixty five percentages of plantlets were well established for *A. saccata* and sixty percentages for *A. cathcartii* in the poly house condition. After one month, regenerated plants were successfully transferred to the field.

The protocol optimized here is efficient, reproducible and provide a rapid technique for mass propagation and multiplication of this two potential medicinal plants and could further be used in their improvement programme.

Figure 3. Growth of plantlets of two species of *Aristolochia* in Poly-house condition. **A** = *A. saccata*; **B-C** = *A. cathcartii.*

Table 3. Effect of auxins for root induction of *A. saccata* (after 2 weeks in root induction medium).

Auxin concentration (mg l^{-1})		Response (%)	Numbers of roots/shoot	Root length/culture
NAA	**IBA**			
0.1		28.3 ± 2.8	1.8±0.34	2.74±0.07
0.5		63.3 ± 2.8	1.8±0.24	3.08±0.13
0.8		51.6 ± 2.8	2.2±0.44	3.02±0.07
1		53.3 ± 2.8	1.2±0.45	2.80±0.04
	0.1	41.6 ± 2.8	1.4±0.55	2.84±0.04
	0.5	83.3 ± 2.8	2.8±0.44	3.22±0.16
	0.8	51.6 ± 2.8	2.4±0.44	3.18±0.10
	1	43.3 ± 2.8	1.6±0.55	2.98±0.11

Data mean of 3 replicates ± S.D.

Table 4. Effect of auxins for root induction of *A. cathcartii* (after 2 weeks in root induction medium)

Auxin concentration (mg l^{-1})		Response (%)	Numbers of roots/shoot	Root length/culture
NAA	**IBA**			
0.1		31.6 ± 2.8	1.8±0.45	2.36±0.11
0.5		56.6 ± 2.8	2.4±0.54	2.42±0.13
0.8		46.6 ± 2.8	2.6±0.55	2.80±0.12
1		43.3 ± 2.8	2.2±0.45	2.82±0.10
	0.1	63.3 ± 2.8	2.8±0.44	2.86±0.08
	0.5	78.3 ± 2.8	3.8±0.44	3.04±0.08
	0.8	66.6 ± 2.8	3.6±0.54	3.02±0.10
	1	56.6 ± 2.8	3.2±0.44	2.92±0.10

Data mean of 3 replicates ± S.D.

4. DISCUSSION

Clonal propagation through tissue culture can be achieved in a short time and space. Thus, it is possible to produce plants in large numbers starting from a single individual protoplast to different plant parts as an explant. Micro propagation has, wide commercial application, starting from conservation of genetic stock of threatened species to secondary metabolite production in important plant taxa and year round supply of disease free quality planting material for commercial cultivation. Since then, several crop species have been micropropagated and recipes are now available which can be adopted by growers trained in aseptic manipulations in a new era of plant husbandry. The results obtained in our experiment suggested that *in vitro* plantlet regeneration using nodal explant may be used for direct clonal propagation and conservation with a low risk of generating disease free quality planting material in large scale for *Aristolochia saccata* and *Aristolochia cathcartii*.

In this study, an *in-vitro* propagation protocol has been developed for *A. saccata* and *A. cathcartii* using nodal explant. In both the plants nodal explants showed direct somatic embryogenesis when cultured on MS medium using various concentrations of BAP (1.0-4.0) and 2iP (1.0-4.0) separately or in combination with low concentration (0.5 and 1.0 mg l^{-1}) of auxin (NAA). It was observed that BAP in combination with NAA was more effective for shoot induction than the hormones used separately.

Among the different treatments of BAP and NAA, 3.0 mg/l BAP + 1.0 mg/l NAA showed better response in case of *A. saccata*. In this concentration, 96.6% explants induced to develop shoots. The number of shoot as well as length of shoot per explant was recorded as 3.4 ± 0.55 and 3.38 ± 0.11 cm respectively. BAP is considered one of the most useful cytokinins for the multiplication of axillary buds reported by many authors [10-12]. In the present investigation, combination of BAP with NAA was found more suitable than BAP and 2iP alone. But, highest shoot length was observed in low concentration of BAP i.e., 4.34 ± 0.07 cm.

Induction of callus and multiple shoots from *A. bracteolate* using various PGRs was also reported previously [13]. Among the BAP - NAA supplemented media for *A. cathcartii*, the best response was achieved in 4.0 mg/l BAP + 0.5 mg/l NAA after 30-day of culture and 88.3% explants showed proliferation in this combination. The highest mean number of shoot per culture were 6.2 ± 0.44 in combination of BAP and NAA, but the highest shoot length was found 4.02 ± 0.1 in 2.0 mg/l BAP. These results are in agreement with the results of Sultana and Handique, Chandramu et al., Sudha et al. and Chen et al. [14-17]. In the present study, it was found that the number of shoot per culture was increased with the number of subculture. Rout et al. (2000) reported that, a rapid rate of propagation depends on the sub-culturing of proliferating shoots [18].

The MS medium augmented with auxin or cytokinin alone or in combinations induced highest percentage of shoot proliferation and maximum number of shoots from the inter-nodal segments of *A. bracteata* [19]. The results of the present study were directly coincided with previous observations [20-25]. In *A. tagala*, multiple shoot buds are produced directly from nodal explants cultured on basal medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA [26] and adventitious shoots at 1.0 mg/l of BAP [27]. *A. bracteolate* cultured on MS medium fortified with 4.0 mg/l of BAP combined with 0.5 mg/l of NAA produce maximum number of shoots (8.9) in nodal explants [28]. In the present study we optimized a protocol for large scale multiplication of *A. saccata* and *A. cathcartii* using nodal segments as explants.

In the present study, nodal explants of *A. saccata* and *A. cathcartii* showed significantly higher response in the medium with the combination of BAP + NAA. The quality of shoots and the overall growth response in terms of average shoot length was better in this growth regulator combination. A comparatively lower response was recorded when BAP or 2iP was added alone in the medium. Review of literature indicates that the addition of either IAA or NAA in the culture medium improved the response in a number of species in terms of shoot growth. Shin et al. [29] reported that the combination and interaction of BAP and NAA plays important role for *in vitro* propagation of nodal explant for multiple shoot induction.

In this study, two cytokinins were taken for higher shoot multiplication. Some authors also suggested that the combination of two cytokinins were needed for producing multiple shoots on *Aristolochia bracteolate* [13], but here higher response was observed in combination of cytokinin and auxin for both the species of *Aristolochia.*

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil [30]. The auxins NAA and IBA were used singly to induce rooting from *in vitro* raised shoot lets. A range of concentration was tested (0.1, 0.5, 0.8 and 1.0 mg/l) for rooting. In the present study 1/2 strength MS basal medium and the two different auxins (NAA and IBA) were tried, the maximum results on rooting were obtained on half strength with IBA (0.5 mg/l) then NAA. The auxins, NAA and IBA were used singly to induce rooting from *in vitro* raised shootlets of *Dioscorea hispida* [31].

The well rooted plants were transferred to plastic cups containing soilrite for hardening and kept under controlled condition. Upon transferred to vermiculite medium plants started producing fresh shoots and roots after one week of transplanting. Later they were transferred to the field and the survival rate was 65% in case of *A. saccata* and 60% in case of *A. cathcartii*. The efficient micro propagation technique described here may be highly use full for raising quality planting material of *A. saccata* and *A. cathcartii* for commercial and off season cultivation which is not only help the ex-situ conservation but also help full in the restoration of genetic stock of the species.

Study of *in-vitro* propagation produced an efficient protocol for large scale multiplication and *ex situ* conservation of the medicinally important plant, *A. saccata* and *A. cathcartii* using nodal segments. It can also be used as a source of tissues for the biochemical characterization of medicinally active compounds and will increase the opportunities for the use of this medicinal plant in both traditional and modern medical health care. Wild medicinal plants are being depleted rapidly due to over-exploitation and unscientific methods of collection. Hence, in the present work, protocol for *in vitro* regeneration of the rare and endemic medicinal plant species *A. saccata* and *A. cathcartii* have been developed. These protocols could be used to make these plants available throughout the year for traditional healers, pharmaceutical usages, germplasm conservation, commercial cultivation, and also for the production of secondary metabolites.

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AUTHORS' CONTRIBUTION

All the authors contributed equally for the success of this research. The final manuscript has been read and approved by all the authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

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