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In vitro Regeneration of Plantlets from Nodal Explants of *Aristolochia bracteata* Retz. – An Important Medicinal Plant

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ABSTRACT: Natural propagation of *Aristolochia* species is hindered by its low viability of seeds. *Aristolochia bracteata* Retz. is one of the major species of the genus that faces challenges with its seed viability. So, its multiplication through the *in vitro* culture is one of the best ways to overcome such challenges. Accordingly, an efficient regeneration protocol through *in vitro* direct organogenesis was developed for the valuable medicinal plant *Aristolochia bracteata Retz.* using nodal segments as explants. The present study established a reproductive protocol for the conservation via *in vitro* culture as well as its secondary metabolite enhancement. Varying concentrations of plant growth regulators of cytokinin (Benzyl adenine, Kinetin and Thidiazuron) and auxin (Indole-3-acetic acid, Indole-3-butyric acid and 1-Naphthaleneacetic acid) were used for micropropagation in Murashige and Skoog medium. The plant showed multiple roots and shoots at lower concentrations of Indole-3-acetic acid (0.9 mg/l) and Benzyl adenine (0.7 mg/l) respectively. Maximum shoots were attained in Thidiazuron, but all the cultures showed anomalous growth. Shoot with large-sized leaves were obtained in kinetin. Rooted plantlets were transferred to the natural environment after appropriate acclimatization.

Abbreviations: BA, Benzyl adenine; KIN, Kinetin; TDZ, Thidiazuron; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; NAA, 1-Naphthaleneacetic acid; MS, Murashige and Skoog.

Keywords: *Aristolochia bracteata*, Murashige and Skoog, Organogenesis, Thidiazuron, Acclimatization.

INTRODUCTION

It is impossible to imagine the survival of humans and animals if the Earth had no plants. Plants are now considered an indispensable source for treating and preventing various diseases. So, it formed the basis of sophisticated traditional medicine systems. In India, around 2500 plant species are being used as part of the indigenous system of medicine (Srivastava *et al*., 1996). *Aristolochia* is a noticeable genus in the family Aristolochiaceae which has extensive significance in traditional medicine (Che *et al*., 1984). *Aristolochia bracteata* Retz. (syn. *Aristolochia bracteolata* Lam.) belongs to them is commonly called "worm killer" in English due to their intended anthelminthic activity and trypanocide effect (Samia *et al*., 2006). "Aduthinnapala" is their local name. The plant was traditionally used by the community of Jubek state in South Sudan for malaria (Mathew *et al*., 2021). The plant was broadly used in Ayurveda. Their leaves have antiulcer, amenorrhoea, anthelmintic (Roy *et al*., 2009) and antiplasmodial activity (Ahmed *et al*., 2010). Leaf decoction in a dose of 50ml is used to treat dysmenorrhea. Dried, powdered roots were used to increase the uterus contractions during labour. Bark decoction or whole plant with a dose of 50 ml is applicable in treating intestinal worms. Powdered leaves are applied in maggot-infested wounds (Tomar, 2017). It also treats lung inflammation, dysentery, and snakebites (Negi *et al*., 2003). Leaves paste is applied externally for snake bites by the villagers of Kumaragiri Hills of Salem district in Tamil Nadu (Alagesaboopathi, 2009). The plant is commonly employed in bacterial infections (Palanisamy *et al*., 2019). It is used to treat malaria with a reported antiplasmodial activity (Mathew *et al*., 2020).

Aqueous shoot extract of *Aristolochia bracteolata* can formulate spherical shaped silver nanoparticles from silver nitrate (Thanh *et al*., 2022). Silver nanoparticles were biosynthesized from *Aristolochia bracteolata* Lam. by Sreeharsha *et al.* (2020). Silver nanoparticles from *Aristolochia bracteolate* showed significant larvicidal and pupicidal efficiency on youngster (I-IVth instar larvae and pupa*) Culex quinquefasciatus mosquito vector* (Narayanan *et al.,* 2022). The phenotypic characteristics of human gingival-derived mesenchymal stem cells (HGMSCs) on induction with total methanol extract of *Aristolochia bracteolata* have been studied by Murugan *et al*. (2017).

The plant possesses anti- inflammatory (Negi *et al*., 2003; Thirumal *et al*., 2012; Nandhini *et al*., 2017), anthelmintic, antipyretic, purgative and abortifacient properties (Kirtikar and Basu 1988). Methanolic root

bark extracts possess anti-oxidative and antidiabetic activities (Agada *et al*., 2022). Petroleum ether plant extracts also possess antimycobacterial activity (Nivedhitha and Indumathy 2022). Aristolochic acid, aristolactam, magnoflorine, ceryl alcohol, β- sitosterol and N- acetylnornuciferine were the utmost chemical constituents reported in *Aristolochia bracteata* Retz. (The Ayurvedic Pharmacopoeia of India, 2008). Aristolochic acid act as a competitive inhibitor of the enzyme phospholipase A2 presented in snake venom (Viswanath *et al.*, 1987). Aristolactam exhibits many pharmacological activities including antioxidant and neuro-protective activities (Costa *et al*., 2013; Jung *et al*., 2009). Methanolic leaf extracts of *Aristolochia bracteolata* promotes the healing of the liver cells via anti-proliferative, antioxidant and anti-inflammatory effects by maintaining the hepatocellular membrane integrity (Gabriel *et al*., 2022).

The genetic diversity and luxuriance of medicinal plants are endangered at an alarming rate due to urbanization, forest degradation, and overharvesting to produce medicines (Sarma & Tanti 2015). Agriculture invasion is also responsible for the extensive degradation of such plant species. Hence, there is a need for sustentation and sustainable usage of such medicinally important plants for future use. The extended germination period and the type of morphophysiological dormancy of seeds have a commutable value for the survival and *in-situ, ex-situ* conservation of *Aristolochia* species (Nakonnechnaya *et al*., 2018). Therefore, the present study aimed to develop a reproductive protocol for conserving *Aristolochia bracteata* Retz. through micropropagation by using nodalex plants.

MATERIALS AND METHODS

Explant selection and sterilization. The plants collected from Tamil Nadu were established in the green house of F.M.N. College, Kollam as a source of explant. They were authenticated by the herbarium of JNTBGRI, Thiruvananthapuram, Kerala (TBGT). Healthy and disease-free shoots were selected for the culture. The explants were treated with 10% labolenefor15minutes and thoroughly washed with running tap water for 30minutes. Then, 0.1% mercuricchloride (w/v) was given for1minute as a final treatment of the sterilization procedure. After that the explants were rinsed with autoclaved double distilled water thrice.

Media preparation and inoculation. MS medium (Murashige and Skoog 1962) was used for the *in vitro* culture of *Aristolochia bracteata* Retz. The required quantity of stock solutions was pipetted out into a beaker containing 0.3% sucrose and 100mg/l myoinositol. Medium amended with different phytohormones were subjected to autoclave for 20 minutes at 121ºC and 15 psi pressure only after adjusting the pH to 5.8 and adding 0.08% agar for solidification of media.

The use of nodal explants permits an adequate multiplication rate, achievable by either shoot segmentation or bud proliferation (Capuana *et al*., 2022). Nodes were dissected out under strict aseptic conditions inside a laminar air flow chamber, which was sterilized by ceaseless exposure to germicidal UV rays for half an hour before use. Autoclaved glass ware sand instruments were used to carry out all the operations. Explants were inoculated to the MS medium fortified with different plant growth regulators. Auxins and cytokinins were the most common plant growth regulators used in *in vitro* cultures for plant tissues (Eudes *et al*., 2003). Cytokinins *viz*., BA, KIN, TDZ and auxins viz.IAA, IBA, NAA were respectively the shooting and rooting plant growth regulators used for the culture. The cultures were incubated at 12/12hour photoperiod under illumination from white fluorescent tubes with 3000 lux light intensity at 25 ± 1ºC.

Regeneration of plantlets and *invitro* rooting. Basal medium fortified with different concentrations of BA (0.5-2mg/l), KIN (0.5-2 mg/l) and TDZ (0.01-0.1 mg/l) were used for *in vitro* shoot regeneration. Regenerated *in vitro* shoots were cultured on MS medium supplemented with different concentrations of rooting hormone. IAA (0.5-2mg/l), IBA (0.5-2mg/l) and NAA (0.5-2mg/l) were used for rooting. The effect of IAA and IBA combined with KIN for the explant regeneration is also studied. Each plant's response regarding the number of shoots and roots was recorded after45 days of inoculation. Data are statistically analysed by the standard error of the mean. *In vitro*-grown plantlets were separated from the culture bottles and washed with double distilled water to remove all traces of the medium. After satisfactory developments of roots, plantlets were triumphantly transplanted into small plastic pots. The plantlets were exposed to sunlight for acclimatization and were maintained in the garden.

RESULT AND DISCUSSION

***In vitro* shoot regeneration from nodal explants.** The plant growth regulator is in dispensable in controlling the plant tissues biological process. Nodal segments regeneration potential were explored on MS medium augmented with various plant growth regulators are summarised in Table 1-3. Nodal explants cultured on MS medium amended with different concentrations of BA, KIN and TDZ developed healthy shoots. Sebastinraj and Sidique (2011) reported shoot multiplication was higher in nodal segments of *Aristolochia bracteata* Retz. All the cultures of BA and KIN were found to accelerate shoot bud differentiation. However, BA was more efficient than KIN in terms of shoot multiplication. Among various concentration of BA, 0.7mg/l showed the highest frequency of shoot regeneration with a response percentage of 100 having 11.75 ± 0.490 shoots shown in Fig. 1 (A). Benson and Jose (2023) also reported that a lower concentration of BA induces multiple shoots in *Aristolochia krisagathra*. Multiple shoots with large leaves were observed on MS medium supplemented with 2mg/l KIN (1.875 ± 0.226) and had 75% growth response. Cytokinin is essential to break the apical dominance in buds and inducing subsidiary meristem grown into shoots (Singh *et al*.,1994). Multiple shoots were induced from relatively low concentrations of TDZ with an anomaly of stunted shoots as in Fig. 2(A). However, TDZ reduces

regeneration potential and results in stunted shoots as suggested by Huetteman and Preece (1993). Multiple shoots and roots were obtained from a combination of KIN and IAA but, the medium supplemented with IBA (1mg/l) in combination with KIN (2 mg/l) produced elongated shoots in Fig. 2 (C).

***In vitro* rooting.** Auxins at low concentrations can induce roots rather than the higher concentration (Lidia *et al.,* 2007). Among the various auxins used, 0.9 mg/l IAA (Fig. 2.B) proved foremost for rooting of *Aristolochia bracteata* Retz. with 37.125 ± 0.833roots showed a 100% growth response. IAA is more effective than IBA for rooting. The varying concentration of NAA has no more effect in rooting, while KIN, combined with IAA and IBA, produced a minimum number of roots.

Acclimatization stage. The achievement of any micropropagation system depends on the potentiality to transfer plantlets from out of the cultures to ambient conditions with an elevated survival rate. The direct transfer of *in vitro*-raised plants to the field induces high mortality (Deb and Imchen 2010). Accordingly, *in vitro* plantlets were removed from the culture bottles and thoroughly washed with running tap water and distilled water to remove all the residues. The plantlets were transferred to autoclaved soil and covered with light plastic covers with sufficient holes to prevent explant mortality and allow light penetration to promote photosynthesis. The plastic covers were removed after acclimatization of the plant to the natural environmental conditions and successfully established in the green house of FMN College, Kollam (Fig. 2D).

Table 1: Effect of cytokinin's (BA, KIN and TDZ) for the shoot multiplication of *Aristolochia bracteata* Retz.

Hormonal concentration (mg/l)			Percentage of response	No. of shoots (Mean ± SE)
BA	KIN	TDZ		
0.5	-	-	100%	5.375 ± 0.943
0.6	-	-	100%	7.875 ± 0.515
0.7	-	-	100%	11.75 ± 0.490
0.8	-	-	100%	8.125 ± 0.515
0.9	-	-	100%	5.875 ± 0.295
1.0	-	-	100%	5.625 ± 0.460
1.5	-	-	87.5%	2.875 ± 0.440
2.0	-	-	62.5%	0.625 ± 0.182
-	0.5	-	50%	0.50± 0.188
-	0.6	-	62.5%	0.625 ± 0.182
-	0.7	-	50%	0.50 ± 0.188
-	0.8	-	75%	0.75 ± 0.163
-	0.9	-	62.5%	0.625 ± 0.182
-	1.0	-	75%	0.875 ± 0.226
-	1.5	-	87.5%	1.125 ± 0.226
-	2.0	-	100%	1.875 ± 0.226
-	-	0.01	100%	13.625 ± 0.532
-	-	0.02	100%	14.75 ± 0.365
-	-	0.03	100%	16.50 ± 0.681
-	-	0.04	100%	18.25 ± 0.590
-	-	0.05	100%	20.875 ± 0.548
-	-	0.06	100%	22.875 ± 0.295
-	-	0.07	100%	23.125 ± 0.440
-	-	0.08	100%	25.875 ± o.666
-	-	0.09	100%	26.125 ± 0.290
-	-	0.10	100%	28.125 ± 0.226

Table 2: Effect of auxins (IAA, IBA and NAA) for the *in vitro* rooting of *Aristolochia bracteata* Retz.

Hormonal concentration (mg/l)			Percentage of response	No. of roots (Mean ± SE)
IAA	IBA	NAA		
0.5	-	-	100%	16.625±0.564
0.6	-	-	100%	24.875 ± 0.742
0.7	-	-	100%	30.5 ± 0.462
0.8	-	-	100%	35.375 ± 0.375
0.9	-	-	100%	37.125 ± 0.833
1.0	-	-	100%	33.125 ± 0.440
1.5	-	-	100%	20.75 ± 0.365
2.0	-	-	100%	17.125 ± 0.350
-	0.5	-	0%	0
-	0.6	-	0%	0
-	0.7	-	75%	1.25 ± 0.313
-	0.8	-	75%	2.625 ± 0.595
-	0.9	-	87.5%	4.125 ± 0.639
-	1.0	-	87.5%	5.125 ± 0.811
-	1.5	-	100%	18.125 ± 0.295
-	2.0	-	100%	21.75 ± 0.365
-	-	0.5	0%	0
-	-	0.6	0%	0
-	-	0.7	0%	0
-	-	0.8	0%	0
-	-	0.9	0%	0
-	-	1.0	0%	0
-	-	1.5	0%	0
-	-	2.0	0%	0

Fig. 1. A. Habitat of *Aristolochia bracteata* Retz. **B.** Initiation of shoot **C.** Shoot multiplication in BA (0.7 mg/l) **D.** Shoot with large sized leaf in 2 mg/l kinetin

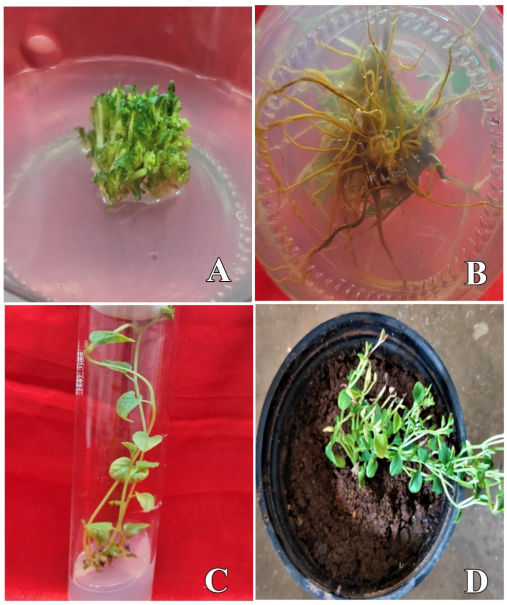

Fig. 2. A. Micro shoots produced in TDZ **B.** Multiple roots in IAA (0.9 mg/l) **C.** shoot elongation in KIN(2mg/l) and IBA (1mg/l) **D.** Acclimatised plant.

Table 3: Effect of IAA and IBA in combination with KIN for the micropropagation of *Aristolochia bracteata* Retz.

Hormonal concentration (mg/l)			Percentage of response	No. of shoots (Mean ± SE)	No. of roots (Mean ± SE)
KIN	IAA	IBA			
2.0	0.5	-	100%	3.125 ± 0.350	2.25 ± 0.453
2.0	0.6	-	100%	1.5 ± 0.188	2.0 ± 0.327
2.0	0.7	-	100%	1.875 ± 0.295	2.25 ±0.365
2.0	0.8	-	100%	1.25 ± 0.163	2.125 ± 0.295
2.0	0.9	-	100%	1.375 ± 0.263	2.375 ± 0.532
2.0	1.0	-	100%	1.5 ± 0.267	2.5 ± 0.462
2.0	1.5	-	100%	2.0 ± 0.377	2.75 ± 0.453
2.0	2.0	-	100%	1.875 ± 0.295	3.25 ± 0.674
2.0	-	0.5	0%	0	0
2.0	-	0.6	0%	0	0
2.0	-	0.7	75%	0.875 ± 0.226	2.125 ± 0.350
2.0	-	0.8	87.5%	1.125 ± 0.226	2.50 ± 0.422
2.0	-	0.9	87.5%	1.375 ± 0.323	2.75 ± 0.411
2.0	-	1.0	87.5%	1.875 ± 0.515	3.25 ± 0.526
2.0	-	1.5	87.5%	1.25 ± 0.225	2.875 ± 0.398
2.0	-	2.0	75%	1.0 ± 0.267	3.125 ± 0.398

CONCLUSIONS

The low viability of seed impedes the natural propagation of the plant. As a part of plant propagation, the present study exemplifies a triumphant development of *in vitro* propagation of *Aristolochia bracteata* Retz. It is known that lower concentration of IAA and BA induced multiple roots and shoots respectively. BA is strongly recommended than kinet into obtain the highest percentage of regeneration and the highest number of shoots without any anomalies. Plantlet with elongated shoots were given from medium amended with a combination of KIN and IBA. However, TDZ produces multiple stunted shoots in which the regenerated plantlet is not taken for further *in vitro* culture studies. In conclusion, the micropropagation method is valuable for conserving *Aristolochia bracteata* Retz., a medicinally important plant. Retardation in its natural propagation can be overcome through this regeneration protocol, and it enhances the overall conservation of the species.

FUTURE SCOPE

The present investigation suggested a reproductive protocol for the micropropagation of *Aristolochia bracteata* Retz., which provides a future conservation strategy for the species. The clones produced rapidly through the *in vitro* method can overcome the meager viability of seeds. The study forms the basis for producing useful secondary metabolites to humans, especially in pharmaceutical science.

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Conflict of Interest. None.

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