Research in Biotechnology, 2(6): 44-52, 2011 ISSN: 2229-791X
www.researchinbiotechnology.com

**Regular Article**

# *In vitro* propagation of *Aristolochia bracteata* Retz. - A medicinally important plant

**Sahaya Sathish S[1], Janakiraman N[1], Johnson M[2*]**

[1]Department of Botany, St. Joseph's College (Autonomous), Tiruchirappalli - 620 002,
[2]Department of Plant Biology and Plant Biotechnology, St. Xavier's College (Autonomous), Palayamkottai - 627 002, Tamil Nadu, India
*Corresponding author email: ptcjohnson@gmail.com

**A direct and indirect propagation system has been established for the medicinally important plant *Aristolochia bracteata* Retz. using inter-nodal segments as explants. The surface sterilization of *A. bracteata* inter-nodal segments was carried with different concentration of mercuric chloride such as 0.05%. 0.1% and 0.15% for different time duration. Among them, 0.1% mercuric chloride for 2 min showed low percentage contamination and highest (96%) percentage of microbes free explants. The explants were cultured on Murashige and Skoog's medium augmented with different concentrations and combinations of plant growth regulators for direct and indirect regeneration. Highest percentage of callus induction (82.3 ± 0.57) from inter-nodal segments was observed on Murashige and Skoog's medium supplemented with 1.5 mg/L of 2,4-D. Three types of calli viz., friable, semi-friable and compact calli were observed from the inter-nodal segments. Highest frequency of shoot proliferation (61.5 ± 0.43) was observed in Murashige and Skoog's medium augmented with 1.0 mg/L of 6 - Benzyl Amino Purine in combination with 1.0 mg/L α - Naphthalene Acetic Acid. Maximum number of shootlets regeneration (2.6 ± 0.15) was also observed from the same medium. The *in vitro* derived calli were sub-cultured for shoot regeneration. The Murashige and Skoog's medium fortified with 1.0 mg/L of 6 - Benzyl Amino Purine in combination with 0.5 mg/L of α - Naphthalene Acetic Acid showed the highest percentage (73.2 ± 0.43) shoot proliferation from the inter- nodal segments derived calli. The *in vitro* raised shootlets were sub-cultured on ½ strength Murashige and Skoog's medium augmented with various concentrations of IAA and IBA for root formation. Highest percentage, maximum number of rootlets/shootlet and mean length of rootlets were observed in ½ Murashige and Skoog's medium supplemented with 1.0 mg/L of IBA. Sixty eight percentages of plantlets were established in the earthen pots.**

**Key words:** *Aristolochia bracteata*; *In vitro*; Calli; Inter-nodal; Organogenesis.

*Aristolochia bracteata* Retz. (Aristolochiaceae) is commonly known as 'Worm killer' in English and 'Aadutheendaapaalai' in Tamil. In the indigenous system of medicine, the decoction of the leaves is used for treating skin diseases and rheumatism (Anjaria *et al*., 2002). It is bitter, purgative, anthelmintic, useful in fevers, painful joints and applied to sores to kill maggots. The leaves are applied to navel to move the

bowels of children and are also given internally in combination with castor oil as a remedy for colic. Bruised leaf mixed with castor oil is applied externally in obstinate cases of eczema of the legs of children (Kirtikar and Basu, 1980). It has been used for the treatment of round worm infection (Nadkarni and Nadkarni, 1954). The root and leaves are bitter, acrid, thermogenic, cathartic, anti-inflammatory, antibacterial, antiperiodic and useful in validated conditions of constipation, foul ulcers, syphilis, gonorrhoea, dyspepsia, skin disorder, eczema and intermittent fevers (Warrier *et al*., 1994, Joy *et al*., 1998). It is also used in traditional medicine as a gastric stimulant and in the treatment of cancer, lung inflammation, dysentery and snake bites (Negi *et al.,* 2003). *A. bracteata* possess aristolochic acid which has various biological activities which includes abortifacient, antiadenocarcinoma, antiaggregant, antibacterial, anticancer, anticarcinomic, antiedemic, antieicosanoid, antifertility, antiherpetic, antiimplantation, antiinflammatory, antineoplastic, antiprostaglandin, antiseptic, antithrombic, antitonsilitic, antitumor, antivaccinia, antiviral, carcinogenic, contraceptive, hemangionagenic, mutagenic, nephrotoxic, papillomagenic, pesticide, phagocytotic, hepatotoxic and immunostimulant properties (URL: http://www.ars-grin.gov/cgi-bin/duke/chem-activities.pl). Due to their medicinal importance, many scientific studies have been carried out on the phytochemical, pharmacological values of *A. bracteata.* Greater demand for plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats (Boro *et al*., 1998). *In vitro* propagation has been proved to be very efficient technique for mass propagation of rare and endangered plant species (Fay, 1992). It offers highly efficient tools for medicinal plants useful in the pharmaceutical industry (Li *et al*., 2007). It has also been reliably used as a powerful tool for mass multiplication of medicinally important plant species (Kayani *et al*., 2008, Chitra *et al*., 2009, Singh *et al*., 2009, Ashrafuzzaman *et al*., 2009, Gokhale *et al*., 2009, Xing *et al*., 2010, Kumaraswamy *et al*., 2010, Ghosh *et al*., 2010, Rani *et al*., 2010). A few published reports are available on micropropagation of *A. bracteolata* (Remeshree *et al*., 1994; Sebastinraj and Sidique, 2011). With this knowledge the present study was aimed to produce an effective reproducible and simple protocol for the large scale multiplication of the economically and medicinally important plant *A. bracteata* using inter-nodal segments and inter-nodal segments derived calli as explants for better exploitation.

**Materials and Methods**

Plants of *Aristolochia bracteata* Retz. (Aristolochiaceae) were collected from Kolli Hills, Salem, Tamil Nadu, India. They were grown in the Botanical garden of St. Joseph's College (Autonomous), Tiruchirappalli, Tamil Nadu, India. Young shoots were harvested and washed with running tap water and surface sterilized in 0.05 and 0.1% mercuric chloride for 2, 3 and 5 min. After rinsing 3-4 times with sterile distilled water, internodes were cut into smaller segments (0.5 to 1.0 cm) and used as explants. The explants were placed horizontally (inter-nodal segments) on solid basal Murashige and Skoog (1962) medium supplemented with 3% sucrose, 0.7% (w/v) agar (Hi-Media, Mumbai) and different concentration (0.5-2.0 mg/L) and combination of BAP and Kinetin for *in vitro* regeneration of shootlets. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 15 min. The cultures were incubated at 25 ± 2°C under cool fluorescent light (2250 lux 12 hr/d photoperiod). For rooting, the *in vitro* raised shootlets were transferred to the ½ MS medium augmented with different concentrations of auxins (IAA, IBA and

NAA). Each and every experiment was performed with 20 replicates and repeated twice. For hardening, the *in vitro* raised plantlets were removed from culture, washed thoroughly with tap water planted in small polycups filled with sterile garden soil (3:1), covered by unperforated polybags irrigated with 10 x diluted MS liquid medium and hardened for 4 weeks in a mist chamber before transfer to field.

**Results**

The inter-nodal segments were surface sterilized with different concentration of mercuric chloride such as 0.05%. 0.1% and 0.15% for different time duration. Among them, 0.1% mercuric chloride for 2 min showed low percentage mortality and contamination (92% percentage of microbes' free explants). The explants treated with 0.1% and 1.5% of mercuric chloride for 3 min showed 50- 65% of explants mortality. 0.05% of mercuric chloride for 3 min treated explants showed less percentage of mortality and high percentage of contamination. The explants treated with 0.05% for 4 min and above, 0.1 and 0.15% for 2½ min and above obtained hundred percentages of microbes' free explants with high percentage of explants mortality, high concentration of mercuric chlorides leads the death of the explants (lethal effect). MS medium supplemented with various concentration of auxins (2,4-D and NAA) and cytokinin (BAP) induce callus formation followed by proliferation of multiple shootlets through indirect organogenesis with varied percentage.

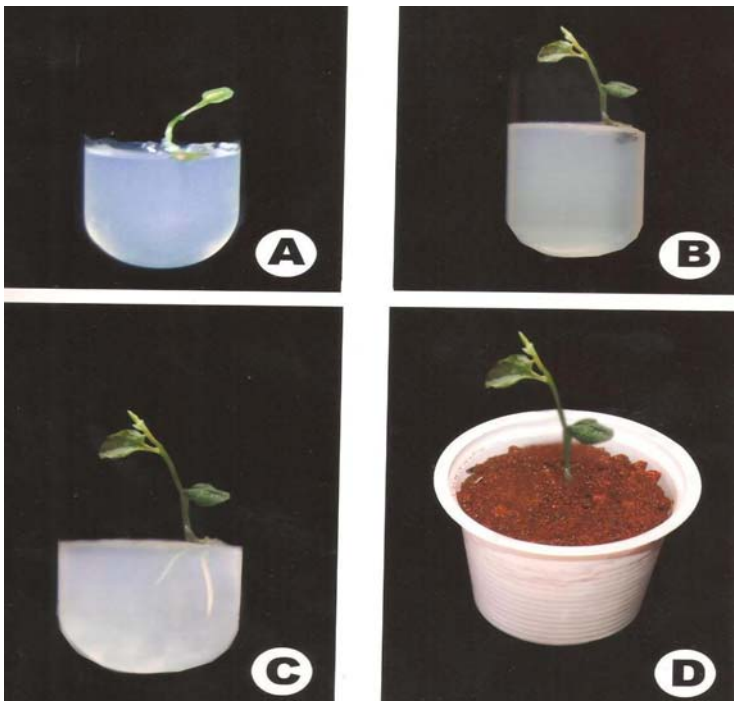


**Fig. 1: Direct organogenesis from inter-nodal explants of *A. bracteata.*** A - Multiple shoots initiation from inter-nodal segments; B - Shoot elongation; C - Rooting of elongated shoots; D - Hardened plantlet

Highest percentage of callus induction (82.3 ± 0.57) from inter-nodal segments was observed on Murashige and Skoog's medium supplemented with 1.5 mg/L of 2,4-D. Three types of calli viz., friable, semi-friable and compact calli were observed from the inter-nodal segments. Highest frequency of shoot proliferation (61.5 ± 0.43) was observed in Murashige and Skoog's medium augmented with 1.0 mg/L of 6 - Benzyl Amino Purine in combination with 1.0 mg/L α - Naphthalene Acetic Acid. Maximum number of shootlets regeneration (2.6 ± 0.15) was also observed from the same medium. The *in vitro* derived calli were sub-cultured for shoot regeneration. According to the plant growth regulators supplementation in the medium explants showed different types callus induction and varied frequency shoot proliferation, the results of the various combinations and concentration of plant growth regulators were tabulated in Table 1. The Murashige and Skoog's medium fortified with 1.0 mg/L of 6 - Benzyl Amino Purine in combination with 0.5 mg/L of α - Naphthalene Acetic Acid showed the highest percentage (73.2 **±** 0.43) shoot proliferation from the inter- nodal segments derived calli. Maximum number of shootlets regeneration (5.5 ± 0.41) and mean length (3.9 ± 0.24) of shootlets was also observed in Murashige and Skoog's medium fortified with 1.0 mg/L of 6 - Benzyl Amino Purine in combination with 0.5 mg/L of α - Naphthalene Acetic Acid (Table 2).

**Table 1: Effect of Auxins and Cytokinin in callus production from inter-nodal segments of *A. bracteata***

| MS Medium + Plant Growth regulators in mg/L | | | % of callus | Type of callus | % of Shoot formation from Inter-nodal segments | Mean number of Shootlets / Inter-nodal segments |
|---|---|---|---|---|---|---|
| 2,4-D | NAA | BAP | | | | |
| 0.5 | 0.00 | 0.00 | 62.3 ± 0.38 | | | |
| 1.0 | 0.00 | 0.00 | 71.2 ± 0.63 | Embryonic | | |
| 1.5 | 0.00 | 0.00 | **82.3 ± 0.57** | Semi- Friable white | | |
| 0.00 | 0.5 | 0.00 | 55.8 ± 0.42 | | | |
| 0.00 | 1.0 | 0.00 | 60.4 ± 0.85 | Green Semi-friable | | |
| 0.00 | 1.5 | 0.00 | 55.6 ± 0.43 | | | |
| 0.00 | 0.00 | 0.5 | 52.3 ± 0.62 | | | |
| 0.00 | 0.00 | 1.0 | 63.8 ± 0.42 | Yellowish White nodular | | |
| 0.00 | 0.00 | 1.5 | 53.6 ± 0.74 | White Semi-friable | | |
| 0.00 | 0.5 | 0.5 | **45.8 ± 0.64** | | 40.8 ± 0.26 | 1.2 ± 0.06 |
| 0.00 | 0.5 | 1.0 | 35.5 ± 0.42 | | 45.4 ± 0.34 | 1.4 ± 0.12 |
| 0.00 | 0.5 | 1.5 | 25.3 ± 0.37 | | 50.6 ± 0.24 | 1.6 ± 0.08 |
| 0.00 | 0.5 | 2.0 | 20.4 ± 0.32 | | 55.8 ± 0.42 | 1.7 ± 0.04 |
| 0.00 | 1.0 | 0.5 | 30.3 ± 0.23 | | 45.3 ± 0.37 | 1.4 ± 0.06 |
| 0.00 | 1.0 | 1.0 | 25.2 ± 0.35 | | **61.5 ± 0.43** | **2.6 ± 0.15** |
| 0.00 | 1.0 | 1.5 | 20.2 ± 0.63 | | 55.3 ± 0.41 | 2.1 ± 0.09 |
| 0.00 | 1.0 | 2.0 | 20.4 ± 0.32 | | 53.8 ± 0.23 | 1.9 ± 0.21 |

**Table 2: Influence of plant growth regulators on Organogenesis of *A. bracteata***

| MS medium with plant growth regulators (mg/L) | | | | % of shootlets formation ± S.E. | Mean length of shootlets ± S.E. | Mean no. of shootlets / explants ± S.E. |
|---|---|---|---|---|---|---|
| BAP | KIN | 2,4-D | NAA | | | |
| 1.0 | 0.0 | 0.0 | 0.0 | 66.5 ± 0.23 | 3.9 ± 0.24 | 4.6 ± 0.34 |
| 1.0 | 0.0 | 0.0 | 0.5 | 73.2 ± 0.43 | 3.2 ± 0.42 | 5.5 ± 0.41 |
| 1.5 | 0.0 | 0.0 | 0.5 | 67.8 ± 0.24 | 3.1 ± 0.34 | 3.2 ± 0.53 |
| 0.0 | 1.0 | 0.0 | 0.0 | 23.4 ± 0.16 | 2.1 ± 0.26 | 1.8 ± 0.35 |
| 0.0 | 1.0 | 0.0 | 0.5 | 35.6 ± 0.35 | 1.8 ± 0.24 | 2.1 ± 0.62 |

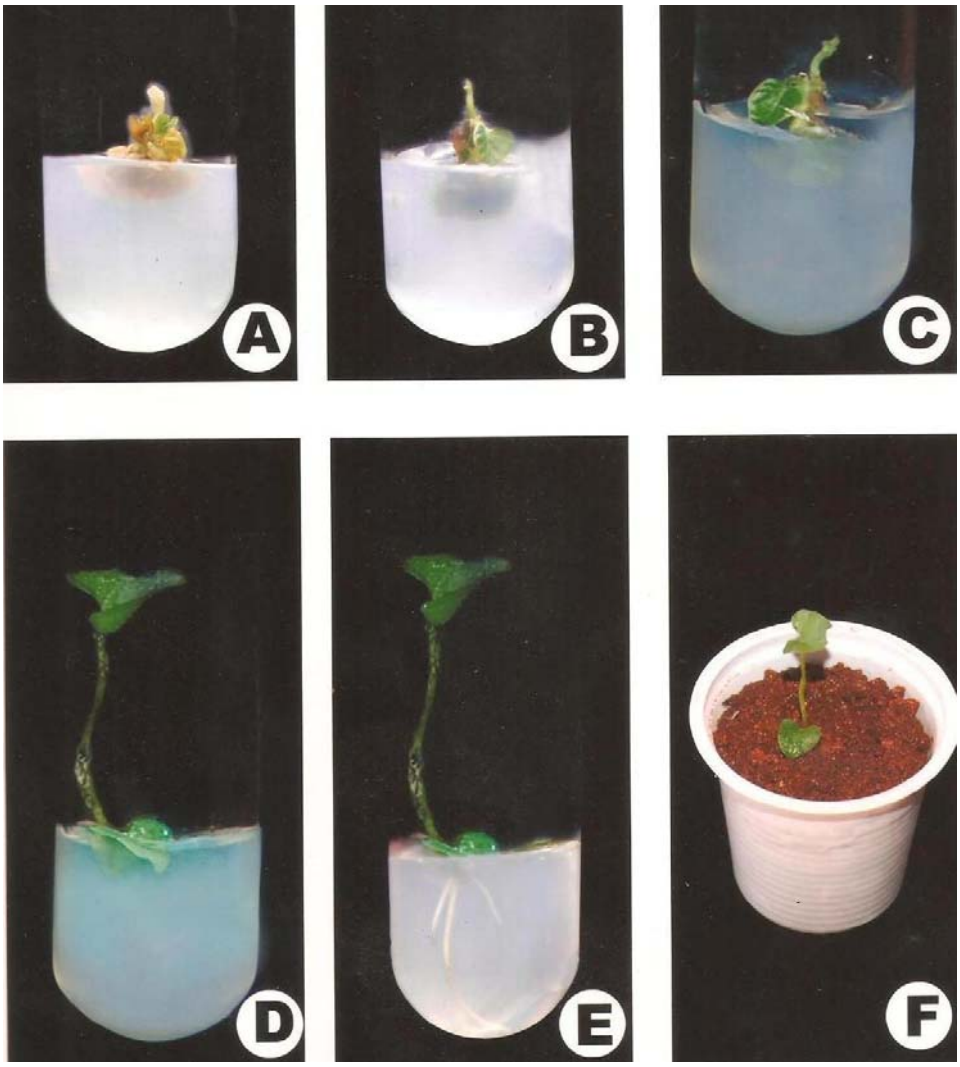


**Fig. 2: Indirect organogenesis from inter-nodal segment derived explants of *A. bracteata.*** A - Callus induction; B - Regeneration of shoot; C - High frequency of shoot formation; D - Shoot elongation; E - Rooting of elongated shoot; F - Hardened plantlet

The *in vitro* raised shootlets were sub-cultured on ½ strength MS medium augmented with various concentrations of IAA and IBA for root formation. At 9th day, the *in vitro* raised shootlets were produced *in vitro* rootlets without any callus proliferation. Highest percentage (75.8 ± 0.64), maximum number of rootlets/shootlet (3.8 ± 0.26) and mean length of rootlets (3.6) were observed in ½ MS medium supplemented with 1.0 mg/L of IBA (Table 3).

**Table 3: Effect of Auxins on rooting on *in vitro* derived shootlets of *A. bracteata***

| Auxins concentration (mg/L) | Mean percentage of rootlets formation ± S.E | Mean no. of rootlets per shootlets ± S.E | Mean length of rootlets in cm |
|---|---|---|---|
| IBA (1.0) | **75.8 ± 0.64** | 3.8 ± 0.26 | 3.6 ± 0.21 |
| IBA (2.0) | 66.6 ± 0.53 | 3.1 ± 0.43 | 2.8 ± 0.34 |
| IBA (3.0) | 57.4 ± 0.46 | 2.3 ± 0.34 | 2.4 ± 0.18 |
| IAA (1.0) | 42.7 ± 0.47 | 1.4 ± 0.24 | 2.1 ± 0.36 |
| IAA (2.0) | 40.3 ± 0.53 | 1.2 ± 0.36 | 2.1 ± 0.16 |
| IAA (3.0) | 35.7 ± 0.42 | 1.1 ± 0.34 | 1.6 ± 0.24 |

After 15 days, *in vitro* raised plantlets were hardened in polycups containing a mixture of sterile garden soil: sand (3:1) covered with polypropylene bags and irrigated with 10 x diluted MS liquid medium. The plants were kept in a culture room for 15 days. Seventy one percentages of plants were successfully established in polycups. After 15 days the polycups hardened plants were transferred to pots and kept in green house. Sixty eight percentages of plantlets were well established in the green house condition. After one month, regenerated plants were successfully transferred to the field.

**Discussion**

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*. The results of the present study were directly coincided with previous observations (Chandra and Bhanja, 2002; Beegum *et al.,* 2007; Kabir *et al.,* 2008; Naika and Krishna, 2008; Chamandoosti, 2010; Kim *et al.,* 2010). 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 (Biswas *et al.,* 2007) and adventitious shoots at 1.0 mg/L of BAP (Chandraprabha and Rama Subbu, 2010). *A. bracteolata* 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 (Sebastinraj and Sidique, 2011). In the present study we optimized a protocol for large scale multiplication of *A. bracteata* using inter-nodal segments as explants. In addition to the direct regeneration, we made an attempt to proliferate the callus induction on inter-nodal segments. The effect of 2,4-D in the induction of callus was reported previously in *Withania somnifera* (Manickam *et al*., 2000), *Rhinacanthus nasutus* (Johnson *et al*., 2005) and in *Phyllanthus amarus* (Johnson, 2007). The inter-nodal segments derived calli were cultured on MS medium augmented with different PGRs induce multiple shoot formation in *Gymnema sylvestris* (Roy *et al*.,

2008), *Vitex leucoxylon* (Chordia *et al.,* 2010) and *Cralluma stalagmifera* (Sreelatha and Pullaiah, 2010). Induction of callus and multiple shoots from *A. bracteolata* using various PGRs was also reported previously (Remeshree *et al*., 1994). In the present study, we also observed highest frequency of shootlets proliferation from the inter-nodal segments derived calli of *A. bracteata* on MS medium augmented with 1.0 mg/L of 6 - Benzyl Amino Purine in combination with 0.5 mg/L of α - Naphthalene Acetic Acid. The results of the present study were directly coincided with earlier observations on *Aristolochia*.

The optimal rootlets formation was observed on half strength MS medium augmented with 0.3 mg/L IBA (Sebastinraj and Sidique, 2011), 1.0 mg/L IBA (Singh *et al*., 2009, Chandraprabha and Rama Subbu, 2010). In the present study also, we observed the highest percentage and maximum number of rootlets per shootlets on half strength medium supplemented with 1.0 mg/L IBA. Hence the results showed consistency with other studies where the addition of IBA promoted the induction of roots in several systems including *Dioscorea zingiberensis* (Chen *et al*., 2003), *Woodfordia fruticosa* (Islam *et al*., 2009) and *Ophiorrhiza eriantha* (Jaimsha *et al*., 2010).

**Conclusion**

The present study produced an efficient protocol for large scale multiplication and e*x situ* conservation of the medicinally important plant *A. bracteata* using inter-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.

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