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Rational development of *in vitro* propagation conservation of worm killer plant (*Aristolochia bracteolata*)

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Abstract
Aristolochia bracteolata Linn. (family- Aristolochiaceae) commonly called as Hukka-Bel and worm killer in English). It is used in traditional medicines as a gastric stimulant and in the treatment of cancer, lung inflammation, dysentery and snakebites. In the indigenous system of medicine, the plant was antipyretic & anti-inflammatory agents. The plant contain Aristolochic acid, has many medicinal properties in various disease condition. The Phytochemical showing revealed the presence of alkaloids, triterpenoids, steroids and sterols, flavonoids, tannins and phenolic compounds and cardio glycosides. Maximum bud break of the explant was obtained on MS medium supplemented with 2.0 mg l^{-1} of benzyl adenine (BA) + additives. Multiple shoots produced from MS+0.25 mgl^{-1} each of BAP, Kinetin and IAA. After 10-15 days of cultures. Amplified shoots from meristematic explants. *In vitro* multiplied shoots pulse treated with root hormone the rooted plantlets were hardened in green house with relieve and transplanted into black polybags containing a 1:1:1 (v/v/v) ratio of garden soil, organic manure and sand.

Keywords: *Aristolochia bracteolata* Micropropagation, *ex vitro* rooting

1. Introduction

Aristolochia bracteolata belongs to the family Aristolochiaceae. There are about 120 species distributed all the way through the tropics and subtropics. *A.bracteolata* commonly called as Hukka-Bel, Juffa and Aulosa and worm killer in English (Bhandari 1978). It is used in traditional medicines as a gastric stimulant and in the treatment of cancer, lung inflammation, dysentery and snakebites. In the indigenous system of medicine, the plant was antipyretic & anti-inflammatory agents. The seeds also contain the same two substances and also a greenish-brown, non-drying fixed oil, an alkaloid is reported present in the root and stem of Indian material (Burkill, 1985, Negi etal.2003). It is proved to have antioxidant property and insecticidal properties. The plant contain Aristolochic acid, has many medicinal properties in various disease condition. The Phytochemical showing revealed the presence of alkaloids, triterpenoids, steroids and sterols, flavonoids, tannins and phenolic compounds and cardio glycosides. The purpose of this study was to develop in vitro techniques for conserving Aristolochia bracteolata by mass multiplication for subsequent reintroduction in their natural habitat as this can be grown in Westland. There is a need to develop micropropagation protocol as plant population has already vanished from the region (Gbadamosi *et al.* 2012).

2. Material and Methods

The stem of Aristolochia bracteolata was cut into two- three cm long nodal segments (with at least one or two node per segments). Nodal shoot explants were first treated with systemic fungicide Bavistin (0.1%) for five-ten minutes and treated with Hgcl2 (0.1 %) for two- four minutes for surface sterilization. These were washed thoroughly with autoclaved water for six-eight times. Surface sterilized nodal shoot explants were inoculated on MS medium (Murashige and Skoog 1962), containing ranges of cytokinin namely BAP (BenzyalaminoPurine) and Kinetin (1.0-5.0 mgl^{-1} each).Explants placed upright on culture medium. Shoots differentiated by axillary bud activation were excised and the mother explants were repeatedly transferred onto fresh MS medium for production of new shoots. In vitro regenerated shoots were cut into shoot segments each with one or two nodes (1.5-2.5 cm) and sub-cultured on MS-basal medium supplemented with auxin IAA (Indole-3-acetic

acid) 0.1 mgl-1 and cytokinins BAP, Kin, 2-iP in different concentrations (0.25-0.5 mgl-1 each for further multiplication. The micro propagated shoots (5-8 cm long) were pulse-treated with different concentrations of IBA (Indole-3-butyric acid) or NAA (α- Naphthalene acetic acid) (100-500 mgl-1 each) for 3 minutes. Shoots treated with root inducing PGRs (Plant growth regulators) were transferred on to bottles containing sterile soil-rite moistened with an aqueous solution of one-fourth strength of MS salts. These bottles were incubated in a greenhouse at 28+2°C. After root initiation the caps of bottles were gradually opened. These were finally removed. The bottles containing plantlets were shifted away from pad section towards fan section. The acclimatized plantlets were transferred to poly-bags containing mixture of garden soil, organic manure and sand (3:2:1). These plantlets were kept in green house for two months and then the hardened and acclimatized plants were shifted to nursery for further evaluation.

3. Results

Juvenile and thick shoot segments with one-two nodes were found to be appropriate for culture initiation. Ninety percent of explants showed bud break within ten days of inoculation. Three-four shoots per explant differentiated on MS medium from each node, supplemented with 2.0 mgl-1of BAP. On Kinetin containing media only one-two shoot regenerated. Out of various concentrations of cytokinins tested, 2.0 mgl-1 of BAP was found to be optimum for initiation of cultures on the MS medium **(Table 1).**

Table 1: Effect of concentrations of cytokines (BAP and Kin) on bud breaking and multiple shoot induction form nodal explants of *Aristolochia bracteolata*

	Concentration (mg l-1)	Response (%)	Shoot number (Mean ±SD)	Shoot length (cm, Mean ±SD)
BAP	1.0	50	1.25±0.43	0.67±0.20
	2.0	80	3.25±0.82	2.77±0.17
	3.0	70	2.50±0.50	1.55±0.42
	5.0	65	1.60±0.80	1.42±0.27
	1.0	25	1.20±0.40	0.96±0.30
	2.0	55	1.75±0.43	1.00±0.60
	3.0	40	1.40±0.48	0.96±0.35
	5.0	35	1.25±0.40	0.55±0.35

The number of shoots increased up to only 2 passages (2-3 shoots), after this on transfer to fresh medium, the shoot number started declining (Fig.-1).

In vitro regenerated shoots were cultured on MS medium supplemented with different cytokinins (BAP, Kin and 2-iP) in different concentrations. Maximum number of shoots (11 to 15 shoots) was produced on MS+0.25 mg l-1 BAP+0.25 mgl-1 + Kin+0.1 mgl-1 IAA+additives, within three-four weeks (Fig. 2).

Multiple shoot induction in on *Aristolochia bracteolata* MS medium +BAP 0.25+Kin 0.25+IAA 0.1 mg l-1

About eight fold rate of shoot multiplication was achieved on 0.25 mgl-1 BAP+0.5 mgl-1 I Kin and 0.5 mgl-1 Kin+0.5 mgl-1 2-iF proved less effective for shoot multiplication as compared to BAP, Kin and IAA. Shoot length and shoot number decreased on MS medium supplemented with 0.5 mgl-1 BAP+0.5 mgl-1 Kin+ mgl-1 IAA+additives (Table 2).

Table 2: Multiplication of shoots of as affected by combination of plant growth regulators

Combinations of PGR's mg l-1	Shoot number (mean ±SD)	Shoot length (Cm, mean ±SD)
BAP 0.25+Kin0.5	9.75±1.40	4.10±0.70
Kin 0.5+ 2-iP 0.5	10.70±1.35	4.20±1.50
BAP 0.25+Kin0.25+IAA 0.1	14.70±2.50	8.50±1.60
BAP 0.5+Kin0.5+IAA 0.1	8.50±1.10	5.70±1.20

it was observed that IBA (500 mgl-1) was best for root induction Cent percent rooting was observed when shoots were treated with 500.0 mgl-1 IBA for 3.0 min. The same percentage of rooting could be treatment of 200ppm IBA but, the number of roots declined. Shoots treated with 100.0 mgl-1 treatment could induce rooting only 90% of shoots (Table - 3).

Table 3: Effect of concentrations of Auxins (IBA and NAA) on ex vitro rooting of micro propagated shoots of *Aristolochia bracteolata*

	Concentration (mg l-1)	Response (%)	Shoot number (Mean±SD)	Shoot length (cm, Mean ±SD)
IBA	100	90	4.25±0.80	0.80±0.20
	200	90	6.30±0.80	2.50±0.30
	500	100	8.00±0.70	3.25±0.82
	100	50	1.90±0.80	1.10±0.20
	200	65	2.95±0.80	2.30±0.82
	500	75	3.40±0.40	2.80±0.30

The rooted plantlets were hardened in green house (Fig.3). The hardened plantlets were finally transferred to polybags. These plantlets were kept in green house for 30-40 days, then shifted to

Nursery and finally transferred to the field for evaluation. Thousands of plants were hardened and field transfer. Hardened and acclimatized plants of *Aristolochia bracteolata* ready for field trials

4. Discussion

Tissue culture techniques are being progressively used for clonal multiplication and in vitro as well as ex vitro conservation of germplasm. (Bonga *et al.* 2010) Young nodal shoots are produced from green house or field was used as ex plants. These were cultured aseptically to induce axillary bud break. Due to induction of basal dormant meristematic tissue of mother explants, repeated transfer was an efficient technique for rejuvenation and reinvigoration and to increase shoot number of in vitro cultures, which has been adopted by many workers on different plants (Panwar *et al.* 2012; Phulwaria *et al.* 2012; Patel *et al.* 2014). Multiple shoots were produced by axillary buds and meristems. The shoots proliferation was done on MS medium supplemented with 2.0 mgl^{-1} BAP. Multiple shoots produced from MS+0.25 mgl^{-1}each of BAP, Kinetin and IAA. After 10-15 days of cultures. Amplified shoots from meristematic explants. *In vitro* multiplied shoots pulse treated with root hormone (IBA). IBA is very effective in promoting rooting of a wide variety of plants, and it is used commercially to root many plant species worldwide (Epstein and Luduig-Muller 1993). About 80% of shoots rooted in green house. The rooted plantlets were hardened in green house with relieve and transplanted into black polybags containing a 1:1:1 (v/v/v) ratio of garden soil, organic manure and sand. The plants in polybags were transferred to the nursery after 40 days of hardening in green house. The in vitro derived plants were phenol typically similar to parental stock and no morphological abnormalities have been observed in the micropropagated plants. This protocol imparts a thriving and quick method for the commercial propagation as well as ex situ conservation of this tropical shrub species.

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