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Induction of *In-Vitro* Flowering and Callogenesis in *Blepharis maderaspatensis* L. (Acanthaceae), A Medicinal Plant

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Abstract: Explants from node, internode and leaf of *Blepharis maderaspatensis* L. were cultured on MS medium supplemented with various growth regulators for *in vitro* callogenesis, organogenesis and flowering. MS medium with 4.53µM 2, 4-D was most effective for callus induction from leaf explant. Flowering was induced *in vitro* when axillary shoots developed on MS medium (with 2.85 µM IAA) were subcultured on MS medium fortified with 8.06 µM NAA and 5.71 µM IAA. 75% of plants survived in field conditions.

Key Words: *Blepharis maderaspatensis*, Callogenesis, *in vitro* rooting, propagation

Introduction

Blepharis maderaspatensis L. is a small pubscent herb with wiry prostrate stem, rooting at nodes, elliptic to obovate leaves in whorls of four, flowers white with yellow spots on lower lip. *Blepharis* is a genus belonging to family Acanthaceae. *Blepharis* is an Afro-asiatic genus comprising 129 species which occur in arid and semi-arid habitats. They are used for the treatment of a number of ailments (Neelambika and Leelavathi 2014). The plant is a major ingredient of many therapeutic ayurvedic compounds. Leaves are used for cuts and wounds. Seeds are diuretic and aprodisiac, used in dysuria and neurotic diseases (Udayan and Balachandran 2011). The leaf paste applied to the forehead is reportedly used to relieve head ache. Juice extracted from leaf of *B. maderaspatensis* is heated with gingelyoil and applied topically on affected places to heal wounds (Ayyanar and Ignacimuthu 2009). Leaf juice is used in throat troubles and asthma (Shanmugam *et al.,* 2009). Leaf is ground into a paste and applied or taken orally to treat bone fracture and deep cuts (Subitha *et al.,* 2011). Whole plant is used to treat urine problems (Marthur and Joshi 2013) and for wound healing (Sundaresan and Senthilkumar 2013).

Owing to the presence of bioactive compounds like allantonin, apigenin, betaine, blepharine, sitosterol and terniflorin, the plant has been used for the production of many drugs (Parotta 2001). *In vitro* developmental protocols are essential to meet the demand for the raw plant material in the market, and thus the plant is selected for the present study. The main objective of this study was to establish a protocol for callogeneis from nodal, internodal and leaf explants and direct shoot multiplication, *in vitro* rooting and flowering from nodal explants.

Materials and Methods

The plant material is originally collected from Mannarghat (Malapuram District, Kerala) and maintained at Calicut University Botanical Garden (Fig.1). Nodal, internodal and leaf explants of *B. maderaspatensis* grown in the Calicut University Botanical Garden (CUBG) were used for the present study. The explants were washed in running tap water (10 min.) followed by a wash in 5% Teepol for five minutes, and a thorough wash with distilled water, surface sterilization with 0.1% $HgCl_2$ for 10 minutes and repeated wash with sterile distilled water. Surface sterilized explants were blot-dried and cultured on MS medium (Murashige and Skoog 1962) containing varying concentrations of auxins and cytokinins, either singly or in combination. Sucrose (3%) was used as the carbon source and agar (0.8%) as the gelling agent. pH of the medium was adjusted to 5.7 before autoclaving at 1.06 Kg cm^{-2} (121ºC). The

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cultures were incubated in 16:8 h light: dark period under flourescent light (25 µmol m^{-2} s^{-1} at 25± 2ºC) in the culture room.

Figure 1: *Blepharis maderaspatensis* L. habit

The responses of explants on different media were recorded daily. Rooting was attempted by keeping the basal cut end of healthy shoots 0.5 cm buried on the MS medium with 17.13 µM IAA. *In vitro* flowering was attempted by subculturing *in vitro* derived shoots on MS medium with 8.06 µM NAA and 5.71 µM IAA. Fully developed plants were transferred to small pots containing sterilized sand and soil (1:1) for acclimatization, and the healthy ones were transfered to earthern pots.

Results and Discussion

MS medium having 2.22 µM BA was found most effective for producing axillary buds (Fig. 2A) after 14 days of incubation. Higher concentrations of BA resulted in stunted growth. MS medium with 4.65 µM Kn was optimal for shoot induction in 14 days. Compared to BA, Kn was optimal for shoot induction in 14 days. Compared to BA, Kn containing medium exhibited more shoot elongation. Synergy of BA (4.44 µM) and Kn (2.32 µM) initiated numerous shoot buds from nodal explant but later resulted in stunted growth in 28 days. Axillary bud multiplication is considered to be the most faithful and simple way for the rapid clonal propagation of desired plants (Shu *et al.,* 2003, Tyagi *et al.,* 2004). Cytokinins play a vital role in the mass multiplication of plants through axillary bud. In the axillary bud multiplication of *B. maderaspatensis*, BA was the best cytokinin compared to Kn. Efficacy of BA in clonal propagation through axillay bud initiation and multiplication has been demonstrated in many medicinal plants like *Rauwolfia tetraphylla* (Faisal and Anis 2002), *Kniphofia leucocephala* (McCartan and Staden 2003) and *Calophyllum apetalum* (Nair and Seeni 2003).

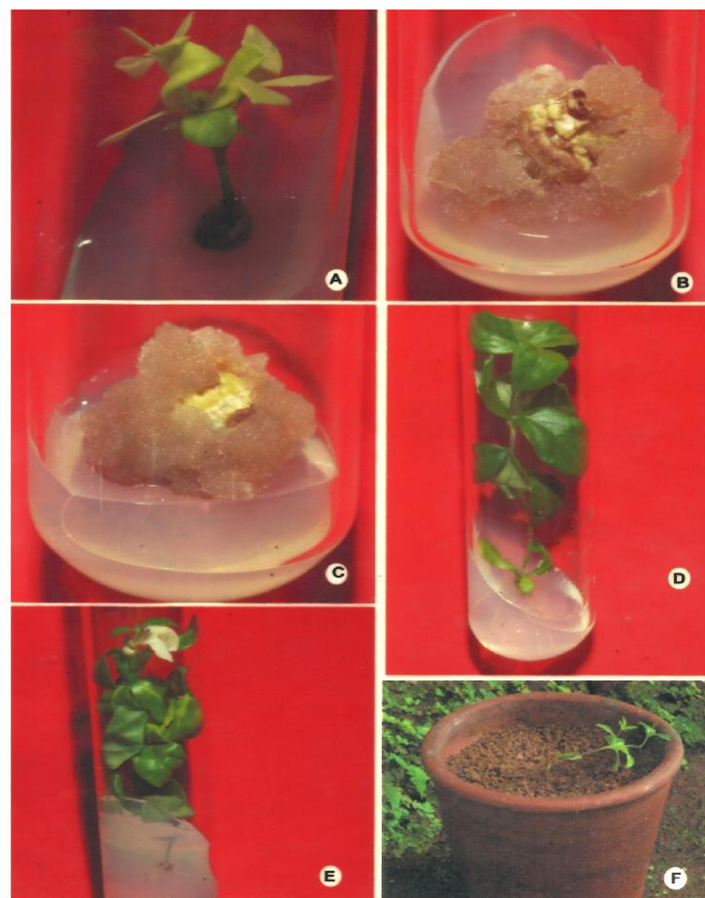

Figure 2: Callogenesis and *in vitro* flowering of *Blepharis maderaspatensis;* A: Axillary bud growth (2.22 µM BA for 35 days); B: Callus from leaf explant (MS medium with 5.37 µM NAA for 25 days); C. Callus from leaf explant (MS medium with 4.52 µM 2,4-D for 15 days) D: *In vitro* root development (sub cultured on MS medium with 17.13 µM IAA for 40 days); E: *In vitro* flowering and rooting of node explant (subcultured on 8.06 µM NAA and 5.71 µM IAA for 35 days) and F: Hardened explant (55 days)

In the present study, a combination of BA (4.44 µM) and IAA (5.71 µM) produced better results. According to Hu and Wang 1983 and Beena *et al.,* 2003, a higher concentration of cytokinins was inhibitory to shoot elongation. An efficient and cost effective protocol for direct regeneration of *Kalanchoe tomentosa*, a valuable medicinal and ornamental plant, was perrformed by Khan *et al.,* 2006. They evaluated the multiplication and growth responses of the plant towards BAP, NAA and a hormone free control MS medium. Mahesh *et al.,* 2012 established an efficient protocol for organogenesis through leaves in *Launaea sarmentosa* (Willd.) Sch. Bip. ex Kuntze, a highly valuable medicinal plant. They found that leaf explants produced microshoots on combination of BAP at 0.5mg/l and NAA at

0.2mg/l in 30 days. Direct and indirect organogenesis was noticed from explants of *Atropa belladonna* by Rani and Prasad 2013. They found under 26±2°C, 14hrs/day 200 lux light, explants with NAA showed direct organogenesis by differentiating into roots. Askari-Khorasgani *et al.,* 2013 evaluated the effects of plant growth regulators on direct regeneration of *Klussia odoratissima*, an endangered medicinal plant with sedative and inflammatory properties. Maximum rate of shoot multiplication was induced when petiole explants were incubated with 2 mg/l BAP and 0.1 mg/l NAA. Ghimire *et al.,* 2010 conducted same study using *Withania somnifera*. They noticed that the regeneration medium that induced the highest numbers of shoots in the petiole and leaf explants was Murashige and Skoog (MS) medium supplemented with 2 mg/l N BA alone or with NAA. Direct regeneration and indirect scalp induction of *Curculigo latifolia* were studied by Babaei *et al.,* 2014 and obtained more number of shoots from shoot tips grown on MS medium supplemented with different concentrations and combinations of thidiazuron and indole-3-butyric acid.

The internodal explants did not develop callus, but leaf segments were better for callogenesis. MS medium fortified with BA (6.66 µM) induced offwhite callus after 7 days of incubation. MS medium with NAA (5.37 µM) also induced calli after 7 days of incubation (Fig. 2B). Of the different growth regulators used, 2, 4-D (4.52 µM) was most effective for callus induction (Table 1; Fig. 2C) which was friable and offwhite. Nodal and leaf explants induced callus on MS medium supplemented with growth regulators. As emphasized by de Klerk *et al.,* 1997, callus is an outcome of mitosis and the trigger for mitosis is probably an auxin. In this study, BA induced callogenesis in leaf and nodal explants. Maximum callus proliferation was observed at 6.66µM BA on leaf explants. Cytokinins triggered callogenesis has also been reported in medicinally important plant, *Lavendula viridis* (Dias *et al.,* 2002). Source of the callus i.e., the explant type, displayed an important role in callus induction, as in the present study; internode did not facilitate induction of callus. However, MS medium supplemented with 4.52µM 2, 4-D was optimum for the callogenesis of *B. maderaspatensis*. 2, 4-D was found giving best results for callogenesis. Similar reports have been available on medicinal plants like *Zanthoxylum stenophyllum* (Binod 2004).

Table 1: Different aspects of callus induction from different explants of *B. maderaspatensis* on MS medium with various growth regulators (µM).

Growth Regulators					% of responses	
BA	Kn	2,4-D	NAA	IBA	Leaf	Node
6.66					60	60
	4.52				80	60
	4.52	16.11			80	40

Table 2: Effect of growth regulators (µM) on *in vitro* rooting from node explant of *B. maderaspatensis* on MS medium.

Growth Regulators			% of responses
IAA	NAA	IBA	
17.13			100
	10.74		80
		4.90	60
2.85	16.11		80

Adventitious roots were developed *in vitro*, upon subcultuing the *in vitro* derived shoots on MS medium supplemented with IAA (17.13µM) (Table 2; Fig. 2D). Adventitious root formation of the *in vitro* propagated shoots determines the success of plantlet survival in field conditions. According to de Klerk *et al.,* 1997, auxins play a major role in *in vitro* rooting. MS medium supplemented with 17.13µM IAA was superior for the induction of roots on *B. maderaspatensis*. Efficacy of IAA in root induction has also been reported in several plants (Zhang *et al.,* 2004). When IAA (2.85 µM) was applied in combination with 16.11µM NAA, higher number of roots were developed from the basal cut end of the node with simultaneous profuse callusing. The *in vitro* rooting studies were also reported in many medicinal plants. Haque and Ghosh 2013 first reported the establishment of micropropagation protocol as well as cytological studies of *Bacopa chamaedryoides* (Kunth) Wettst., an important Indian ethno-medicinal herb. They also used the nodal segments as explants and *in vitro* rooting of multiplied individual shoots was achieved on half strength MS medium supplemented with 50% of 'Aloe vera gel', with a maximum of 18.3 ± 0.17 roots. Up to 66.7% of these multiplied shoots induced healthy flowers *in vitro* on MS medium containing low concentration of 6-benzyl-aminopurine (0.2 mg L−1). *In vitro* root induction and its antibacterial activity of *Costus igneus* was also conducted by Nagarajana *et al.,* 2011. They used two growth regulators IAA and IBA in combinations with MS medium for direct root induction. *In vitro* rooting was also studied using the plant *Bacopa monnieri* by Mohanta

and Sahoo 2014. They found for rooting MS+ Agar 7 g/l, Sugar 20g/l and MS+Agar 8g/l to be the best medium for rooting.

When shoots developed from nodal explants (on MS medium with 2.85µM IAA) subcultured on MS medium in combination with NAA and IAA, flowering and rooting were induced simultaneously in 28 days. A combination of 8.06µM NAA and 5.71 µM IAA gave the best results (Fig. 2E). According to Handro 1983, low concentration of auxin is beneficial for the induction of *in vitro* flowering. Chithra *et al.,* 2003 reported *in vitro* flowering in *Rotula aquatica* using NAA and silver nitrate. *In vitro* flowering and effective micropropagation protocol were studied in *Swertia chirayita*, an important medicinal plant using axillary bud explants by Sharma *et al.,* 2014. Incubation of flowering cultures on BAP supplemented medium was found necessary for flowering within 6 weeks. Aileni *et al.,* 2011 described an efficient protocol for the induction of *in vitro* flowering and fruiting in *Scoparia dulcis*, a multipurpose folk medicinal plant. MS medium supplemented with kinetin and indole-3-acetic acid is found optimal for the formation of multiple shoots, which also induced floral buds.

For acclimatization in the room conditions, well rooted shoots were transferred to small pots filled with pre-sterilized mixture of fertile soil and sand (1:1), then healthy ones were transplanted to earthern pots and maintained in the green house of CUBG (Fig. 2F). Over 75% plantlets survived upon acclimatization.

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